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INTERAÇÃO DE BACTÉRIAS BENÉFICAS ASSOCIATIVAS (*Herbaspirillum seropedicae* e *Azospirillum brasilense*) COM DIFERENTES ESPÉCIES DE GRAMÍNEAS (*Zea mays*, *Brachypodium distachyon* e *Setaria viridis*)

Florianópolis

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Tese submetida ao Programa de Pós-Graduação em Recursos Genéticos Vegetais da Universidade Federal de Santa Catarina, para obtenção do Grau de Doutor em Ciências.
Orientador: Profª. Drª. Ana Carolina Maisonnave Arisi

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Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível.

Charles Chaplin

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RESUMO

O nitrogênio é um nutriente essencial no crescimento das plantas. A habilidade da planta em suprir a quantidade de nitrogênio necessária pode vir da fixação biológica de nitrogênio (FBN), através de interações com bactérias diazotróficas associativas. *Azospirillum brasilense*, que coloniza a rizosfera, e *Herbaspirillum seropedicae,* capaz de colonizar tecidos internos, são exemplos de bactérias benéficas que se associam com raízes de gramíneas de importância agrícola e econômica. O processo de interação planta-bactéria envolve diversos processos que ainda não estão totalmente elucidados.

No presente trabalho foram avaliados parâmetros de crescimento e a regulação transcricional de 10 genes em plântulas de milho (*Zea mays*) inoculadas com *H. seropedicae* SmR1 1, 4, 7 e 10 dias após inoculação (DAI). Foi observado um aumento no número de raízes laterais das plantas com 7 e 10 DAI. Aumento significativo, foi observado na quantificação de transcritos dos genes *Zmko1* e *ZmrbohC* em raízes das plantas inoculadas com *H.seropedicae* aos 4 DAI. Deste modo concluímos que *Herbaspirillum seropedicae* SmR1 presente nas raízes de milho pode alterar a expressão do gene *Zmko1* que esta envolvido na biossíntese de giberelina bem como do gene *ZmrbohC* envolvido na resposta de *burst* oxidativo, no inicio da interação.

Brachypodium distachyon tem sido proposto como sistema modelo de plantas C_3, pois possui atributos atrativos como, por exemplo, baixa estatura, curto ciclo de vida e genoma sequenciado. A fim de selecionar um sistema modelo de gramíneas para estudos de interação planta-bactéria, foi realizada uma seleção utilizando 23 acessos de *Brachypodium distachyon* inoculados com *Azospirillum brasilense* e *Herbaspirillum seropedicae* crescidos em condições de baixo nitrogênio e/ou sem nitrogênio. Nos parâmetros de crescimento, 4 genótipos do tratamento sem nitrogênio e 3 genótipos tratados com baixo nitrogênio, responderam a pelo menos um dos parâmetros de crescimento avaliados em *B. distachyon*, A colonização externa e dos tecidos internos das raízes das plantas, foi visualizada utilizando cepas de bactérias marcadas. Deste modo podemos concluir que as respostas aos parâmetros de crescimento em plantas de *B. distachyon* em associação com estas bactérias, são altamente variáveis quanto ao genótipo, no entanto *B. distachyon* é fortemente colonizada por *A.brasilense* e *H. seropedicae*.

Investigamos as respostas fisiológicas e metabólicas relacionadas ao processo de interação planta-bactéria, além de medir o nitrogênio fixado e assimilado em plantas de *S. viridis* linhagem A10.1 crescidas sob baixo nitrogênio e comparadas a plantas com alta disponibilidade de nitrogênio. Utilizando radioisótopos de vida curta, num primeiro momento $^{11}CO_2$, foi analisada a fixação, distribuição e alocação de CO_2 em plantas inoculadas com *A.brasilense* e *H. seropedicae* crescidas com baixa disponibilidade de nitrogênio, bem como o perfil de açúcares e aminoácidos. Os resultados permitem concluir que plantas de *S. viridis* inoculadas com ambas bactérias e baixa disponibilidade de nitrogênio demonstraram comportamento similar ao de plantas supridas com nitrogênio para os parâmetros analisados. Por fim, utilizando $^{13}N_2$ demonstramos evidências de nitrogênio fixado em plantas de *S. viridis* inoculadas com *A.brasilense* e *H. seropedicae* crescidas em condições de baixo nitrogênio.

ABSTRACT

Nitrogen is an essential nutrient in plant growth. The ability of the plant to supply the required amount of nitrogen can come from biological nitrogen fixation (BNF), through interactions with diazotrophic bacteria. *Azospirillum brasilense*, which colonize the rhizosphere, and *Herbaspirillum seropedicae*, able to colonize the internal tissues, are examples of beneficial bacteria that associate with roots of grasses with agricultural and economic importance. The plant - bacterium interaction involves several processes that still unclear.

In this work the growth parameters and transcriptional regulation of 10 genes were evaluated in maize seedlings (*Zea mays*) inoculated with *H. seropedicae* SmR1 at 1, 4, 7 and 10 days after inoculation (DAI). An increase was observed in lateral roots number of the plants with 7 and 10 DAI. Significant increase was observed in the transcripts level of *Zmko*1 and *Zmrboh*C genes in maize roots plant inoculated with *H.seropedicae* at 4 DAI. In conclusion, *Herbaspirillum seropedicae* SmR1 present in maize roots can influence the expression of *Zmko*1 gene that is involved in the gibberellins biosynthesis and also the expression of *Zmrboh*C gene involved in burst oxidative response at the beginning of the interaction.

Brachypodium distachyon has been proposed as a model system of C_3 plants. It has attractive features such as short stature, short life cycle and sequenced genome. In order to select a grass model for plant - bacterium interaction system, we screened 23 accessions of *Brachypodium distachyon* inoculated with *Azospirillum brasilense* and *Herbaspirillum seropedicae*. The plants were grown under no nitrogen or low nitrogen condition. In growth parameters, four genotypes under no nitrogen and three genotypes under low nitrogen condition, showed positive responses at least for one of the analyzed parameters. External and internal roots colonization tissues of plant were visualized using labeled bacteria strains. The growth parameters responses in *B. distachyon* plants associated with *A. brasilense* and *H. seropedicae* were highly variable to the different genotypes, however *B. distachyon* was strong colonized by *A.brasilense* and *H. seropedicae*.

We also investigated the physiological and metabolic responses related to the plant - bacterium interaction process, and measure the fixed nitrogen and photoassimilates in plants of *Setaria viridis* genotype A10.1 grown under low nitrogen and compared to plants grown under high nitrogen availability. Using short-lived radioisotopes, as $^{11}CO_2$, at first was analyzed CO_2 fixation, distribution and allocation in plants inoculated with *A.brasilense* and *H. seropedicae* grown under low nitrogen condition. Furthermore, were analyzed the sugars profile and amino acids pools. The results indicate that plants of *S. viridis* inoculated with both bacteria grown under low nitrogen condition demonstrated similar behavior to the plants supplied with normal nitrogen. Finally, using $^{13}N_2$ was demonstrated evidence of fixed nitrogen in *S. viridis* plants inoculated with *A.brasilense* and *H. seropedicae* grown under low nitrogen condition.

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INTRODUÇÃO

Plantas e microrganismos interagem naturalmente no solo formando uma estreita e complexa rede de comunicação. Esta comunicação entre planta-microrganismo envolve desde sinais bioquímicos a moleculares que podem ser alterados conforme o tipo de associação. Diversos grupos de bactérias são capazes de se associar com plantas, sendo que essas interações podem ser patogênicas e prejudicar o tecido vegetal, ou interações benéficas que se baseiam na troca de favores entre bactéria e planta. Entre as associações benéficas destacam-se as bactérias associativas que fixam nitrogênio, através do processo de fixação biológica de nitrogênio (FBN). Sabe-se que as plantas não são capazes de fixar nitrogênio atmosférico e que este é um dos componentes mais importantes para o seu desenvolvimento e crescimento, por isso a importância da interação com bactérias. Entre os processos de fixação altamente eficientes e bem documentados destaca-se a simbiose entre bactérias do gênero *Rhizobium, Bradyrhizobium e Azorhizobium* e leguminosas (família *Fabacea*), caracterizada pela formação do nódulo onde ocorrem as trocas entre planta e bactéria. Em contraste com esse sistema simbionte, bactérias diazotróficas como *Azospirillum brasilense* e *Herbaspirillum seropedicae* formam relações associativas e/ou endofíticas com diversas plantas incluindo cereais de importância agrícola e econômica como milho, arroz e trigo, sem a formação de qualquer estrutura especializada. Os benefícios da interação vão além da fixação de nitrogênio, estas bactérias são capazes de promover o crescimento vegetal através da produção de hormônios vegetais como auxinas, citocininas e giberelinas, e ainda podem ser exploradas por seu potencial como biofertilizantes.

Recentemente um grande número de estudos envolvendo bactérias diazotróficas (BRUSAMARELLO-SANTOS et al., 2012; CANGAHUALA-INOCENTE, G. C. et al., 2013; COMPANT; CLEMENT; SESSITSCH, 2010; MONTEIRO et al., 2012; PENG et al., 2010; REINHOLD-HUREK; HUREK, 2011; VARGAS et al., 2012; WALKER et al., 2012), tem sido realizados com o intuito de investigar e elucidar processos fisiológicos, metabólicos e genéticos envolvidos no mecanismo de interação entre planta-bactéria. Porém, a maioria utiliza sistemas agrícolas como milho e arroz, que em sua maioria estão voltados para a produção alimentar. Por este motivo torna-se de fundamental importância adotar um sistema modelo para gramíneas que permita investigar os mecanismos e a genética da associação planta-bactéria. Duas espécies pertencentes à família Poaceae, *Brachypodium distachyon*

(VOGEL, J. P. et al., 2010) e *Setaria viridis* (BENNETZEN et al., 2012; BRUTNELL et al., 2010), estão sendo propostas como sistemas de plantas modelo por apresentarem características atrativas como tamanho, estabilidade para transformação e por possuírem o genoma sequenciado.

Esta tese é apresentada em forma de capítulos, sendo que no capítulo 1 apresenta-se uma revisão bibliográfica sobre a importância do nitrogênio como principal componente de crescimento da planta, incluindo o processo de fixação biológica. Conta também com uma descrição sobre o processo de interação e colonização de plantas por bactérias diazotróficas e sua importância. Por fim, são descritas as características das bactérias, *Herbaspirillum seropediceae* e *Azospirillum brasilense*, bem como das plantas *Zea mays*, *Brachypodium distachyon* e *Setaria viridis.* Os seguintes capítulos são apresentados em formato de artigo científico.

No capítulo 2 está apresentado o trabalho sobre a regulação de transcritos envolvidos nos processos iniciais da interação em raízes de milho associadas à bactéria endofítica *H. seropedicae.* O objetivo desse estudo foi investigar se a presença de *H. seropedicae* associada a raízes de milho variedade DKB 240 pode interferir na expressão de genes da planta relacionados ao metabolismo de hormônios vegetais, bem como em resposta a colonização por essa bactéria. Este trabalho foi realizado no Departamento de Ciência e Tecnologia de Alimentos (CAL), no Centro de Ciências Agrárias (CCA) da Universidade Federal de Santa Catarina (UFSC).

O capitulo 3 apresenta o uso do radioisótopo $^{11}CO_2$ administrado em folhas intactas de *Setaria viridis*, um potencial sistema modelo para estudos de interação com bactérias fixadoras de nitrogênio. O objetivo desse estudo foi investigar respostas fisiológicas e metabólicas em *S. viridis* inoculada com *Azospirillum brasilense* e *Herbaspirillum seropedicae* utilizando carbono-11 como $^{11}CO_2$ para análise de fotoassimilados, açúcares e aminoácidos. Este trabalho foi realizado no Bioscience Department, Brookhaven National Laboratory (BNL), New York, Estados Unidos, durante o doutorado sanduiche pelo programa Ciência sem Fronteiras – INCT/FBN, sob supervisão do Dr. Gary Stacey e Dr. Richard Ferrieri.

No capitulo 4 está apresentado evidências de fixação de nitrogênio por *Azospirillum brasilense* e *Herbaspirillum seropedicae* inoculados em plantas intactas de *S. viridis*, através do radioisotopo $^{13}N_2$. O objetivo deste estudo foi medir a fixação e a incorporação de $^{13}N_2$ gasoso, em plantas de *S.viridis* não inoculadas e inoculadas com ambas as bactérias citadas acima. Este trabalho foi realizado no Bioscience Department, Brookhaven National

Laboratory (BNL), New York, Estados Unidos, durante o doutorado sanduiche pelo programa Ciência sem Fronteiras – INCT/FBN, sob supervisão do Dr. Gary Stacey e Dr. Richard Ferrieri.

O capitulo 5 apresenta a seleção de um sistema modelo para estudos de interação planta-bactéria fixadora de nitrogênio. O objetivo desse trabalho foi avaliar a resposta de diferentes acessos de *Brachypodium distachyon* inoculados com *Azospirillum brasilense* e *Herbaspirillum seropedicae,* através de parâmetros de crescimento e técnicas de microscopia. Este trabalho foi realizado, em Divisions of Plant Science and Biochemistry, Christopher S. Bond Life Science Center (BLSC), University of Missouri em Columbia, Missouri, Estados Unidos, durante o doutorado sanduiche pelo programa Ciência sem Fronteiras – INCT/FBN, sob supervisão do Dr. Gary Stacey.

CAPÍTULO 1

REVISÃO BIBLIOGRÁFICA

1 REVISÃO BIBLIOGRÁFICA

1.1 NITROGÊNIO: USO NA AGRICULTURA E EFICIÊNCIA

O solo é um ambiente variável quanto à disponibilidade de nutrientes e quanto a fatores físico-químicos, por isso o uso de fertilizante nitrogenado é de grande importância na produção agrícola (ELBELTAGY et al., 2001).

O nitrogênio é um constituinte primário de nucleotídeos, proteínas e outros componentes celulares. No entanto, a disponibilidade de nitrogênio, tanto quanto de qualquer outro nutriente limita o crescimento das plantas afetando diretamente a produção primária e essencialmente a estrutura e função da maior parte dos ecossistemas. Nos sistemas agrícolas, o nitrogênio é ainda mais importante, uma vez que sua adição, para sustentar e aumentar o rendimento das culturas é uma característica universal e fundamental do manejo da cultura moderna (ROBERTSON; VITOUSEK, 2009). O uso de adubação nitrogenada aumentou aproximadamente 10 vezes nos últimos anos (ESTRADA et al., 2013; ROBERTSON; VITOUSEK, 2009). Embora tenha ocorrido um aumento na aplicação de fertilizantes, a baixa eficiência do uso do nitrogênio, visto que apenas 1/3 é utilizado pelas plantas (PENG et al., 2010), resulta em perdas e impactos negativos para o ambiente. Isto inclui a lixiviação de nitrato no solo, o escoamento superficial de nitrogênio (N) e fósforo (P), eutrofização dos ecossistemas aquáticos, bem como efeitos sobre a diversidade microbiana (ADESEMOYE; KLOEPPER, 2009).

Com a preocupação em amenizar os efeitos negativos de fertilizantes químicos, a agricultura moderna vem adotando práticas sustentáveis para minimizar o impacto no meio ambiente, e também diminuir os custos de produção sem alterar a produtividade. Uma estratégia é explorar o potencial de microrganismos benéficos de plantas como inoculantes agrícolas (LUCY; REED; GLICK, 2004; LUGTENBERG, B.; KAMILOVA, 2009; OKON, Y.; LABANDERAGONZALEZ, 1994) bem como, na aplicação como fitorremediadores (CHERIAN et al., 2012; LUGTENBERG, B.; KAMILOVA, 2009). Entre esses biofertilizantes destacam-se as bactérias associativas promotoras de crescimento vegetal, as quais são capazes de fixar nitrogênio atmosférico através da fixação biológica de nitrogênio (FBN).

1.1.1 Fixação biológica de nitrogênio (FBN)

As plantas não são capazes de absorver nitrogênio atmosférico diretamente, sendo assim, utilizam o nitrogênio disponível no solo na forma de amônio e nitratos, que são absorvidos através de suas raízes. Por outro lado, um pequeno número de microrganismos é capaz de absorver nitrogênio atmosférico através de um processo chamado de fixação biológica do nitrogênio (FBN). Este processo consiste na conversão de N_2 atmosférico em amônia (NH_3), forma na qual pode ser utilizada pelas plantas (FRANCHE; LINDSTROM; ELMERICH, 2009; LAM et al., 1996). Os microrganismos responsáveis pela fixação de nitrogênio são chamados diazotróficos, os quais possuem o complexo da nitrogenase, enzima que catalisa a conversão de N_2 em amônia. O complexo da nitrogenase é altamente conservado em diazotróficos de vida livre e simbiontes (SANTI; BOGUSZ; FRANCHE, 2013). A maioria destes microrganismos, expressa genes de fixação de nitrogênio – *nif*. Estes genes foram detectados em bactérias endofíticas presentes no apoplasto de raízes de gramíneas (REINHOLD-HUREK; HUREK, 2011; RONCATO-MACCARI et al., 2003). Na maioria deles a expressão é dependente de um ativador de transcrição o *NifA*. Estudos prévios mostram que *NifA* desempenha um papel fundamental na regulação da síntese e atividade da nitrogenase em resposta a disponibilidade de amônia e oxigênio em *Azospirillum brasilense* (ARSENE; KAMINSKI; ELMERICH, 1996; LI, H. et al., 2011).

As complexas interações entre planta e bactéria podem ser benéficas, estimulando o crescimento de plantas e melhorando a produção agrícola; ou prejudiciais causando doenças ao hospedeiro (RAMOS et al., 2011). Geralmente, essas interações são mutualísticas com microrganismos benéficos, tais como rizóbios, micorrizas, endofíticos e rizobactérias promotoras de crescimento vegetal (PGPR). Algumas associações com culturas de importância agrícola estão bem elucidadas e são largamente utilizadas como a interação de leguminosas com *Rhizobium* (por exemplo, soja e *Bradyrhizobium*), o qual forma uma simbiose capaz de fornecer nitrogênio suficiente para desenvolvimento da planta e formação de grão (ALVES; BODDEY; URQUIAGA, 2003). Após o reconhecimento de ambos, planta e bactéria, o estabelecimento da simbiose é caracterizado pela formação de um órgão diferenciado, o nódulo, o qual promove um nicho para a bactéria fixar nitrogênio (HUNGRIA, M.; STACEY, 1997; MEILHOC et al., 2011). Outra associação bastante difundida que exige a formação de uma estrutura especializada é aquela que ocorre entre

plantas e fungos micorrízicos arbusculares. Estes fungos ajudam a planta na absorção de água e nutrientes do solo, como fosfato e nitrato e em troca a planta fornece carboidratos. Nesta associação, uma estrutura é formada após a invasão das hifas do fungo nas raízes da planta, o arbúsculo, local onde ocorrem as trocas entre o fungo e a planta (DELAUX et al., 2013; VENKATESHWARAN et al., 2013).

Em contraste com estes simbiontes, bactérias diazotróficas associativas interagem com uma ampla variedade de plantas incluindo cereais de importância agrícola e econômica. Algumas bactérias podem estabelecer associação endofítica, ou seja, são capazes de colonizar espaços internos da planta e, além de fixar nitrogênio, podem proporcionar condições apropriadas para proteger o complexo da nitrogenase à exposição ao oxigênio (SANTI et al., 2013).

Gramíneas não formam associações simbióticas naturalmente com fixadores de nitrogênio, no entanto, tem sido demonstrado que uma parte considerável de seu nitrogênio deriva da FBN. Embora a quantidade de nitrogênio fixado não seja tão grande quanto aquela fixada pelos nódulos de rizóbios associados com leguminosas, em plantas actinorrízicas ou em associações de cianobactérias, um aumento no rendimento tem sido relatado em plantas inoculadas com bactérias promotoras de crescimento vegetal (ESTRADA et al., 2013; PEDRAZA, RAUL O. et al., 2009) incluindo plantas cultivadas no campo (BHATTACHARYYA; JHA, 2012; DOBBELAERE, S.; VANDERLEYDEN; OKON, 2003).

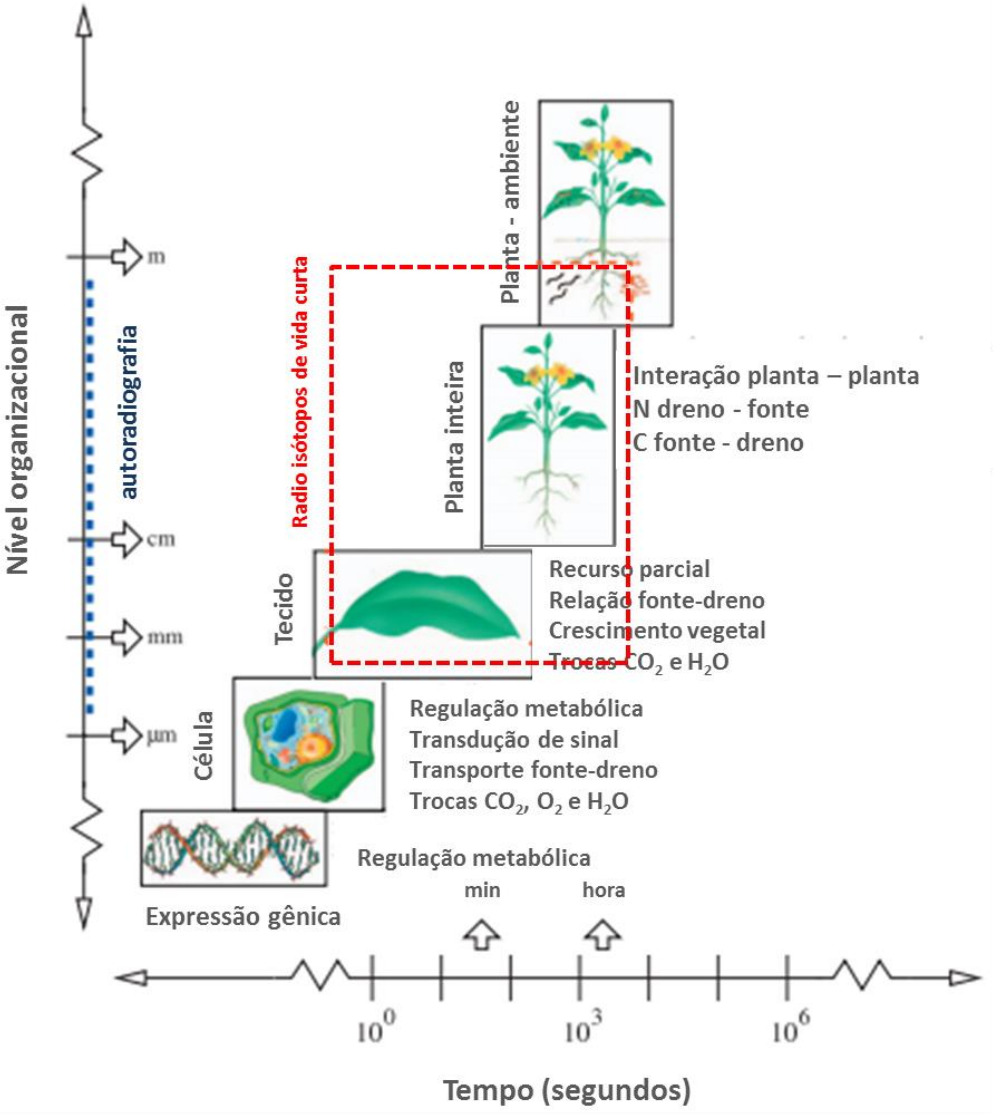
Para demonstrar a capacidade de fixação de nitrogênio pela bactéria, ensaios de redução de acetileno, diluição isotópica de ^{15}N, análise transcricional da expressão entre genes repórteres e *nifH*, e análise de transcritos que codificam o complexo nitrogenase através de qRT-PCR, tem sido utilizadas. Estas técnicas revelam a atividade da nitrogenase em diversas bactérias endofíticas em interação com gramíneas incluindo *G. diazotrophicus*, *Azoarcus sp* e *Herbaspirillum sp.* em arroz bem como rizobactérias do gênero *Azospirillum* em interação com milho (SANTI et al., 2013).

Um método não invasivo, que não danifica a planta, é o uso de radioisótopos instáveis como nitrogênio-13. Nitrogênio-13 ($t_{½}$= 9,97 min) é o único isótopo radioativo de nitrogênio que decai por emissão de pósitrons, o qual possui 9,97 minutos de meia vida (Meeks *et al*., 1978; Caldwell *et al*., 1984). Este radioisótopo proporciona oportunidade única para conduzir métodos não invasivos em plantas intactas, permitindo medir a atividade de fixação de nitrogênio, bem como, a sua redistribuição em tecidos-alvo, em um curto espaço de tempo. O uso deste isótopo tem sido amplamente utilizado em plantas para investigar o transporte e

assimilação de nitrogênio pelas raízes nas formas $^{13}NO_3^-$, $^{13}NH_4^+$ e ^{13}NN. Além disso, nitrogênio-13 permite medir as mudanças no metabolismo de nitrogênio na planta.

O uso de radioisótopos de curta duração, em estudos que envolvem planta inteira pode ajudar a elucidar desde os processos enzimáticos que desencadeiam reações bioquímicas, às respostas fisiológicas das plantas a estímulos ambientais (KISER et al., 2008) (Figura 1.1).

Figura 1.1: Processos temporais e espaciais hierárquicos de crescimento e desenvolvimento da planta. Diferentes processos a nível subcelular e celular vinculados a níveis organizacionais mais altos via de transdução de sinal. No tecido, planta inteira e planta em interação com microambiente, os processos dinâmicos podem ser medidos usando radioisótopos de curta duração com a detecção de raios gama e técnicas de imagem como a autoradiografia. A linha tracejada em vermelho exemplifica regiões onde os radioisótopos podem ser administrados (raízes, planta inteira ou folha).



Fonte: adaptado de Kiser et al., 2008.

Kasel e colaboradores (2010) utilizaram $^{13}N_{2,}$ na forma gasosa, para medir a fixação de nitrogênio em nódulos radiculares de plantas de soja. Em resumo, os autores foram capazes de otimizar o processo de aplicação, e os resultados mostraram quantidades razoáveis de nitrogênio-13, demonstrando que esta técnica pode ser aplicada para estudos de assimilação de nitrogênio em plantas. Hanik e colaboradores (2010) utilizando $^{13}NH_3$ gasoso, em folhas intactas de *Nicotiana tabacum*, investigaram mudanças na utilização de nitrogênio durante o processo de indução de defesa, desencadeada por metil jasmonato. Os autores concluíram que esta técnica permite compreender a utilização de nitrogênio na planta, bem como se torna uma ferramenta importante para estudar o metabolismo das plantas.

1.2 INTERAÇÃO PLANTA-BACTÉRIA

Muitas das bactérias consideradas promotoras de crescimento vegetal são diazotróficas capazes de estabelecer associações com diferentes plantas incluindo importantes cereais e gramíneas como trigo, arroz, milho e cana de açúcar. Existem diferentes tipos de bactérias promotoras de crescimento, aquelas que colonizam a rizosfera e possuem efeitos benéficos às plantas, são chamadas bactérias promotoras de crescimento vegetal (BPCV). Quando apresentam uma estreita associação com as raízes, podem ser chamadas de bactérias associativas fixadoras de nitrogênio enquanto que, são consideradas endofíticas fixadoras de nitrogênio aquelas encontradas nos tecidos internos de plantas (REINHOLD-HUREK, B.; HUREK, T., 1998). Bactérias ou rizobactérias promotoras de crescimento podem estimular o crescimento da planta, aumentar o rendimento das culturas, reduzir infecções por patógenos bem como reduzir estresses bióticos e abióticos sem prejudicar a planta. Podem ainda estimular o desenvolvimento de raízes (OLIVEIRA et al., 2009), raízes laterais e em alguns casos aumento da biomassa de raiz e folha (CRUZ et al., 2001; PEDRAZA, RAUL O. et al., 2009; REIS et al., 2000). Entre os gêneros mais conhecidos estão *Acetobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Glucenobacter* e *Pseudomonas* (BALDANI, J. I. et al., 1996; DOBEREINER, 1992; OLIVEIRA et al., 2009). Entre eles *Azoarcus* spp., *Herbaspirillum seropedicae* e *Glucenobacter* são considerados endofíticos.

1.2.1 Processo de colonização de plantas

Vários carboidratos, aminoácidos, ácidos orgânicos bem como outros compostos que fornecem uma fonte de nutrientes para as bactérias associativas, são liberados na rizosfera como exsudato. Estas bactérias são conhecidas por serem quimicamente atraídos e movimentam-se pelo exsudato da planta, que permite a colonização e multiplicação, tanto na rizosfera quanto no rizoplano (COMPANT et al., 2010). A composição do exsudato é influenciada por diversos fatores como o tipo de solo, a disponibilidade de nutrientes, o genótipo da planta, o estágio de desenvolvimento e a exposição da planta a estresses ambientais bióticos e abióticos. Essas diferenças na composição do exsudato da raiz podem influenciar a colonização (LUGTENBERG, B. J. J.; DEKKERS; BLOEMBERG, 2001). O processo de exsudação é conhecido por ser heterogêneo em espaço, ou seja, alguns compostos liberados estão mais concentrados em algumas zonas da raiz do que em outras. Devido a essa diferença, algumas partes da raiz são mais colonizadas que outras, resultando em diferenças espaciais de colonização bacteriana (GAMALERO et al., 2004).

O processo de colonização envolve a produção de diversos componentes e sinalizadores produzidos tanto pela planta quanto pela bactéria, como por exemplo, flavonoides e antibióticos. Qualquer que seja o tipo de interação bactéria-planta, os flavonoides parecem estar relacionados com a resposta do microrganismo em relação à planta. Na interação simbiótica encontramos as bactérias indutoras de nódulos radiculares pertencentes aos gêneros *Rhizobium*, *Bradyrhizobium* e *Azorhizobium*, entre outros. Estas bactérias são capazes de se associarem em simbiose altamente organizada com a planta hospedeira de leguminosas. Este processo de nodulação é controlado pela troca de sinais entre a bactéria simbionte e a planta hospedeira. Uma grande variedade de flavonoides (chalconas, flavononas, isoflavonas e flavonóis) está envolvida na indução dos genes *nod* (SHAW; MORRIS; HOOKER, 2006). Na interação com bactérias simbióticas, os flavonoides são importantes sinalizadores. Flavonoides como flavonona naringenina foi encontrado regulando genes de *H. seropedicae* envolvidos no processo de colonização (TADRA-SFEIR et al., 2011), bem como, estimulando a colonização de trigo por *Azospirillum brasilense*, e também foi responsável pelo aumento do número de raízes laterais em plantas de *Arabidopsis* colonizadas com *Herbaspirillum seropedicae* (WEBSTER et al., 1998).

Algumas BPCV produzem e secretam antibióticos, os quais são particularmente relevantes para a colonização da rizosfera (VAN LOON, 2007), assim como outros

metabólitos, que lhes permite competir com a microbiota natural da planta, promovendo ainda mais sua capacidade competitiva no ambiente radicular.

Deste modo, pode-se dizer que as bactérias associativas podem empregar uma variedade de mecanismos distintos, isolados ou em combinação, para colonizar com sucesso o sistema radicular da planta.

1.2.2 *Herbaspirillum seropedicae*

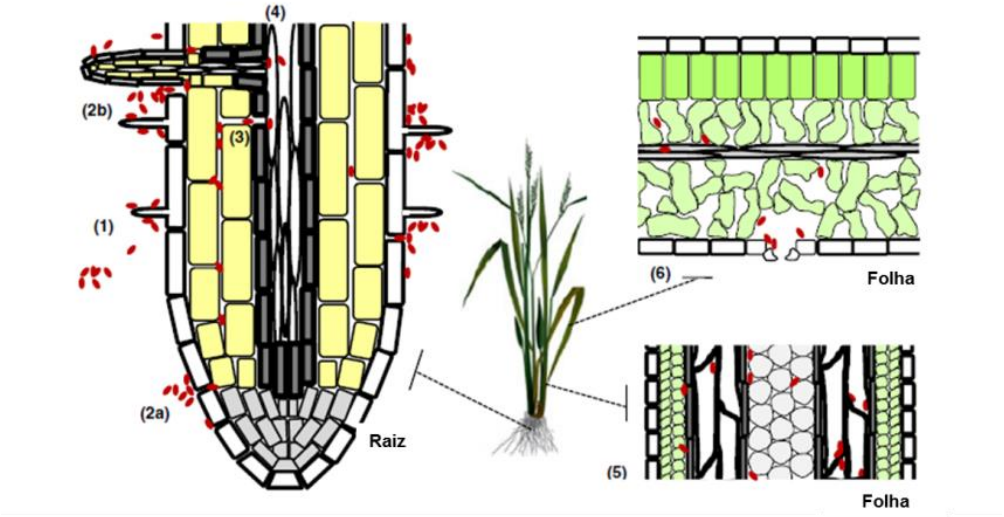
Herbaspirillum seropedicae é uma bactéria diazotrófica endofítica, aeróbia, gram-negativa, fixadora de nitrogênio e promotora de crescimento vegetal. Esta bactéria foi isolada das raízes de milho (*Zea mays*), arroz (*Oryza sativa*), sorgo (*Sorghum bicolor*), cana de açúcar (híbridos interespecíficos de *Saccharum*) e espécies tropicais como a bananeira (*Musa* spp.) e abacaxizeiro (*Ananas comosus*) (BALDANI, J. I. et al., 1986; CRUZ et al., 2001; PIMENTEL et al., 1991). Pertencente à classe das β-proteobacteria, *Herbaspirillum seropedicae* é capaz de colonizar os tecidos internos de plantas de importantes culturas (milho, cana de açúcar, arroz, trigo e sorgo) sem causar qualquer prejuízo à planta hospedeira (PEDROSA et al., 2011). Esta bactéria fixa nitrogênio em condições limitadas de amônio e oxigênio, como foi demonstrado através do ensaio de redução de acetileno e incorporação de $^{15}N_2$ em meio JNFb semissólido sem fonte de nitrogênio (BALDANI, V. L. D. et al., 1992; PIMENTEL et al., 1991). A capacidade desta bactéria de produzir e secretar hormônios vegetais estimula o crescimento da planta, atua na defesa do hospedeiro contra microrganismos patogênicos, e pode ainda influenciar diretamente o aumento da produção de grãos (SCHMIDT et al., 2011).

O papel destas bactérias diazotróficas em associação com gramíneas, principalmente com cereais, foi estudado e progressos foram alcançados em aspectos ecológicos, fisiológicos, bioquímicos e genéticos (BALDANI, J. I.; BALDANI, 2005; MONTEIRO et al., 2012; RAMOS et al., 2011). O genoma de *H. seropedicae* cepa SmR1, uma cepa espontaneamente resistente a estreptomicina mutante da cepa Z78 [15] (ATCC 35893), foi sequenciado e anotado pelo Programa Genoma do estado do Paraná (Genopar Consortium, www.genopar.org), apresentando um único cromossomo circular de 5.513.887 pares de base (PEDROSA et al., 2011).

Monteiro e colaboradores (2012) demonstraram o mecanismo de colonização de *H. seropedicae* em milho (Figura 1.2). A bactéria inicia a colonização pela superfície da raiz, principalmente, nos pontos de emergência de raízes laterais. Após a penetração superficial nos

tecidos da planta, a bactéria espalha-se rapidamente pelos tecidos internos ocupando espaços intercelulares das células da raiz movendo-se para parte aérea através do xilema.

Figura 1.2: Perfil de colonização de *Herbaspirillum seropedicae* em gramíneas.



Fonte: Adaptado de Monteiro et al., 2012.

Todo este processo de colonização inicia com sinais bioquímicos entre a bactéria e a planta, gerando modificações moleculares em ambos. A associação *H. seropedicae* – gramínea não causa prejuízos às plantas hospedeiras, mas, pelo contrário, promove o crescimento vegetal, através da capacidade de produzir compostos que estimulam o desenvolvimento das plantas como, por exemplo, a capacidade da bactéria em produzir fitormônios (BASTIAN et al., 1998).

1.2.3 *Azospirillum brasilense*

Azospirillum brasilense é uma bactéria gram-negativa, fixadora de nitrogênio, pertencente à classe das α – proteobactérias. Isolados da rizosfera de muitas gramíneas e cereais em todo o mundo (*Z. mays, O. sativa, Saccharum officinarum, Sorghum bicolor*) em áreas tropicais, bem como em áreas de clima temperado (DOBEREINER; MARRIEL; NERY, 1976; TARRAND; KRIEG; DOBEREINER, 1978). O gênero *Azospirilla* exibe quimiotaxia com ácidos orgânicos, açúcares, aminoácidos e componentes aromáticos, bem como com exsudatos da raiz. Coloniza, principalmente, a rizosfera de gramíneas de importância agrícola

promovendo o crescimento e aumentando o rendimento de cereais (STEENHOUDT; VANDERLEYDEN, 2000). Estes microrganismos não produzem nódulos, mas podem produzir compostos sinalizadores que estimulam o crescimento de plantas, conferem resistência a doenças e aumentam a mobilização de nutrientes no solo (BARASSI et al., 2007).

Bactérias do gênero *Azospirilla* são capazes de produzir hormônios vegetais como auxina AIA (ácido indol acético), citocininas, giberelinas e etileno, que estimulam principalmente o crescimento radicular das plantas hospedeiras (HUNGRIA, MARIANGELA et al., 2010; SPAEPEN; VANDERLEYDEN, 2011). Estudos demonstram que AIA e citocininas produzidos pela bactéria desempenham um papel central na regulação do desenvolvimento da planta, determinando a arquitetura das raízes. Isto implica no processo de elongação da raiz bem como na formação de raízes laterais (STEENHOUDT; VANDERLEYDEN, 2000). Além da produção de AIA e citocininas, a giberelina produzida por *Azospirillum* está relacionada com os estágios iniciais do desenvolvimento vegetal, estimulando o crescimento do sistema foliar e radicular, influenciando no aumento do número de pelos radiculares (SANTI et al., 2013).

O grande número de informação disponível para este gênero permite concluir que *Azospirillum* possui mecanismos versáteis que combinados afetam o crescimento vegetal. Mesmo sendo considerada uma RPCV, é importante ressaltar que o sucesso de interação com esta bactéria, depende tanto da espécie da planta quanto da bactéria, bem como das condições do ambiente para ocorrer a colonização (SANTI, BOGUSZ E FRANCHE, 2013).

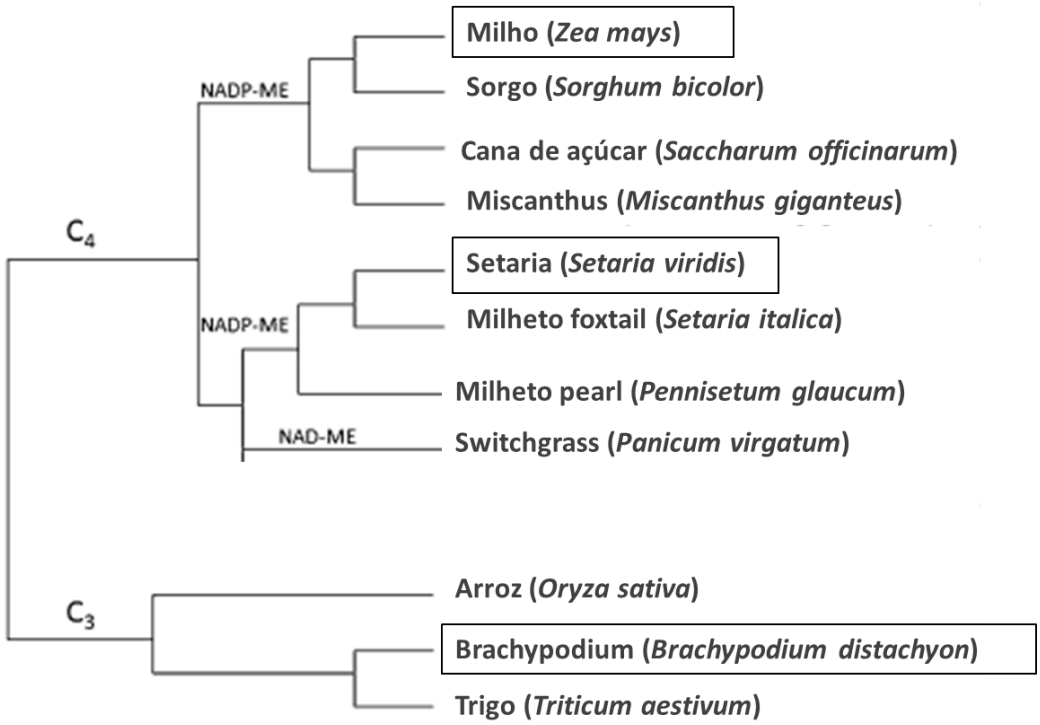
1.3 GRAMÍNEAS E SUA IMPORTÂNCIA

A maior parte da alimentação humana provém de gramíneas, que inclui as principais culturas de cereais bem como gramíneas forrageiras (KELLER; FEUILLET, 2000). A alta produtividade destas gramíneas mostra-se como fonte promissora de energia sustentável.

A família Poaceae compreende mais de 600 gêneros com mais de 10.000 espécies que dominam muitos sistemas agrícolas e ecológicos. A maioria dos estudos de genômica em gramíneas focam em duas subfamílias economicamente importantes: Ehrhartoideae (arroz) e Panicoideae (milho, sorgo, cana e milheto), sendo que arroz, sorgo e milho possuem seu genoma sequenciado e disponível em bancos de dados. Embora arroz e milho sejam considerados modelos de estudos para fisiologia e genética, essas plantas não representam um

sistema de planta modelo como Arabidopsis, pois possuem longo ciclo de vida e considerável tamanho físico para ser mantidas em laboratório. Todas essas características são exemplificadas no que é, sem dúvida, o sistema modelo de planta mais desenvolvida, a Arabidopsis. No entanto, filogeneticamente, a Arabidopsis está distante da família Poaceae. Dentro deste cenário, *Brachypodium* e *Setaria* podem ser alternativas de planta modelo entre as gramíneas. Estas plantas possuem seu genoma sequenciado, baixa estatura e curto ciclo de vida, além disso, têm estreita relação filogenética com importantes cereais (Figura 1.3), o que as torna atrativas como um sistema modelo para o entendimento da biologia e genética de gramíneas.

Figura 1.3: Relações filogenéticas dos membros da família Poaceae, agrupando plantas C_3 e C_4.



Retângulos pretos representam os gêneros investigados neste trabalho.

Fonte: Adaptado de Li e Brutnell, 2011.

1.3.1 *Zea mays*

Além de sua importância agrícola, econômica e alimentar, o milho é também um modelo para estudos de fisiologia e genética de plantas devido à sua alta variabilidade e diversidade genética. Essa espécie tem contribuído significativamente para a elucidação de

processos fundamentais como reprodução, fotossíntese, biossíntese de metabólitos primários. Mais recentemente, investigações utilizando ferramentas genéticas para estudo de hibridização, marcadores assistidos, QTLs, entre outros, as quais geraram uma gama de informações que podem ser exploradas a partir do sequenciamento do genoma da espécie (SCHNABLE et al., 2009).

O milho é uma cultura que remove grandes quantidades de nitrogênio e usualmente requer o uso de adubação nitrogenada para complementar a quantidade suprida pelo solo, quando se deseja melhorar a produtividade. No entanto, o ambiente agrícola e econômico atual busca minimizar a aplicação de fertilizantes nitrogenados para evitar a poluição do ambiente por nitratos, porém, sem perder o rendimento. A compreensão do metabolismo e genética de reciclagem de nutrientes absorvidos durante o crescimento e desenvolvimento da planta, melhorando o uso eficiente do nitrogênio, é dessa maneira, de particular importância para a qualidade da colheita e produtividade, evitando o uso excessivo de fertilizantes (HIREL et al., 2001). Neste sentido a utilização de microrganismos que aumentem a superfície de absorção de nutrientes e fixem nitrogênio em associação com gramíneas, torna-se uma alternativa eficiente para minimizar o uso de químicos nesta cultura.

Rizobactérias diazotróficas do gênero *Azospirillum* e bactérias endofíticas como *Herbaspirillum seropedicae* formam associação com raízes de milho. A colonização dá-se através de sinais bioquímicos (exsudato da raiz) e moleculares liberados por ambos, permitindo o reconhecimento entre a planta e a bactéria. Estudos de transcriptoma (GALLAIS; HIREL, 2004; NESTLER; SCHUETZ; HOCHHOLDINGER, 2011) e proteoma (CANGAHUALA-INOCENTE et al., 2013) vêm sendo conduzidos com esta espécie para elucidação de mudanças no metabolismo e nos mecanismos de resposta a associações com microrganismos benéficos como bactérias promotoras de crescimento.

1.3.2 *Brachypodium distachyon*

Brachypodium distachyon é uma gramínea anual C_3 (via fotossintética), originária do norte da África, sul da Europa, e da Ásia Central, amplamente encontrada em regiões temperadas. Essa gramínea tornou-se atrativa como um sistema modelo para cereais por possuir sintenia genética com trigo, cevada e aveia, por apresentar pequena estatura (15 a 30 cm de altura), tempo de geração curto (8 semanas), genoma pequeno (272 Mbp), capacidade de autopolinização e por ser facilmente cultivada (DRAPER et al., 2001; GARVIN et al.,

2008). Esta gramínea exibe muitas características agronômicas de grande importância nos cereais, como tolerância ao frio, resistência a certos patógenos e mecanismos de dormência (DRAPER et al., 2001). Os diferentes genotipos de *Brachypodium* existentes possuem alta variabilidade quanto ao tempo de florescimento, exigindo um fotoperíodo longo (20h luz). Por serem plantas de clima frio, a vernalização é uma estratégia importante para reduzir a variabilidade e o tempo de florescimento das plantas (BRKLJACIC et al., 2011). A vernalização consiste em expor sementes (para sincronizar a germinação) ou plântulas (para floração) a baixas temperaturas em uma escala de 0°C a 7°C por um tempo prolongado. Com relação ao tempo ideal de vernalização, Schwartz e colaboradores (2010) testando sementes e plântulas de 27 acessos de *Brachypodium distachyon* de diferentes regiões geográficas, mantidas durante 0, 2, 4 e 6 semanas a 4°C, concluiram que longos períodos de vernalização (seis semanas) são ideais para uma efetiva indução de florescimento na maioria dos acessos.

Brachypodium foi inicialmente proposto como um modelo para estudar as interações planta-patógeno, como fungo causador da ferrugem e da brusone em arroz. Recentemente mostrou-se como hospedeiro de espécies de *Fusarium*, principal doença que afeta plantas de trigo (PERALDI et al., 2011). Por outro lado, exibe variações naturais de resistência a fungos patogênicos como *Botrytis cinerea* e *Bipolaris sorokiniana* (POGORELKO et al., 2013), bem como *Puccinia graminis* (FIGUEROA et al., 2013).

Esta gramínea é particularmente útil para estudar sistemas de raízes maduras, pois o sistema radicular de gramíneas difere substancialmente na estrutura e desenvolvimento do sistema radicular de *Arabidopsis*. Ao contrário de raízes de arroz, milho e trigo, os quais apresentam sistema radicular extenso para estudar em condições controladas, as raízes de *Brachypodium* podem ser facilmente analisadas nestas condições (WATT et al., 2009). Além disso, esta planta está sendo utilizada como uma fonte alternativa de produção de biocombustível baseado em biomassa, já que, as principais fontes de energia renovável, soja e milho, estão envolvidos na dieta alimentar humana e animal (HONG et al., 2011; VEGA-SANCHEZ; RONALD, 2010).

Deste modo, todas as evidências acima indicam que *Brachypodium distachyon* é uma planta adequada para investigar mecanismos de resposta de interação entre gramíneas e microrganismos.

1.3.3 *Setaria viridis*

Existem diversos exemplos de gramíneas que podem ser usadas como planta modelo incluindo *Brachypodium distachyon* e arroz (*Oryza sativa*) as quais são atualmente usadas para investigações genéticas (VOGEL, J.; HILL, 2008). No entanto entre as gramíneas existe uma diferença fisiológica relacionada ao mecanismo fotossintético. Arroz e *Brachypodium distachyon* são exemplos de plantas que utilizam a via fotossintética C_3 (fixação de carbono) enquanto que milho, sorgo, switchgrass (*Panicum virgatum*), *Miscanthus* (*Miscanthus giganteus*) e *Setaria* são plantas C_4 (WANG et al., 2009). Entre esses exemplos, *Setaria* está sendo proposta como sistema modelo de planta C_4, pois possui todos os atributos necessários incluindo pequena estatura (10-15 cm), autofertilização e ciclo de vida de 60 dias. Cada planta de *Setaria viridis* é capaz de produzir até 13.000 sementes. Além disso, o sistema de transformação de *S. viridis* já estabelecido por Brutnell e colaboradores (2010), regenerou plantas a partir de calos de sementes, estabelecendo transformações transientes e sistemas estáveis de transformação utilizando *Agrobacterium*.

Setaria viridis é o ancestral da domesticada *Setaria italica* (milheto foxtail), a qual é uma cultura importante que faz parte da dieta alimentar em regiões do norte da China e Índia. *S. italica* possui seu genoma sequenciado pela colaboração entre o Instituto do Genoma (JGI), Departamento de energia dos Estados Unidos (DOE-USA) e Instituto de genômica de Beijing (BGI) (BENNETZEN et al., 2012; ZHANG et al., 2012), enquanto que, *S. viridis* possui seu genoma parcialmente sequenciado. Porém, esforços vêm sendo feitos através do Instituto do Genoma (DOE - JGI Joint Genome Institute) para anotar e sequenciar também essa espécie (BENNETZEN et al., 2012).

Como um modelo C_4 recentemente proposto, muitas informações básicas sobre recursos genéticos e moleculares de *S. viridis* ainda não estão elucidados e, portanto, não estão disponíveis em bancos de dados como, por exemplo, identificação e determinação de sequência de genes. Estudos com transcriptoma vêm realizando uma análise abrangente utilizando RNA de amostras coletadas de diferentes estágios de desenvolvimento e diferentes tecidos de *S. viridis*. Apresentando uma descrição funcional de genes identificados e expressos em *S. viridis*, os dados de transcriptoma encontrados por Xu e colaboradores (2013) permitiram a identificação de um numero maior de repetições de sequencias únicas (SSR). Os transcritos identificados e SSR podem ser utilizados para facilitar futuros estudos de genética envolvendo *S. viridis*.

Coletivamente, essas características sugerem que *S. viridis* é ideal para estudos de evolução de plantas C_4, investigação genômica de gramíneas, e ainda pode ser utilizada como matéria-prima para biocombustíveis (BRUTNELL et al., 2010; LI, P.; BRUTNELL, 2011).

1.4 ANÁLISE DA EXPRESSÃO GÊNICA DIFERENCIAL

Mudanças na regulação da expressão gênica exercem uma profunda influência na morfologia, fisiologia e desenvolvimento de plantas. A mudança no nível de expressão de transcritos de plantas de milho pode ser causada por relações de simbiose com bactérias endofíticas promotoras de crescimento.

A base molecular da interação endofítica vem sendo estudada, mas ainda não está totalmente entendida (REINHOLD-HUREK; HUREK, 2011). A interação planta-bactéria desencadeia mecanismos de defesa que alteram o metabolismo da planta gerando um processo de sinalização que dá inicio a diversas reações bioquímicas que estimulam a expressão diferencial de genes (TADRA-SFEIR et al., 2011; TAO et al., 2009). Estes processos incluem as vias de biossíntese de fitormônios produzidos tanto pela planta quanto pela bactéria e, possuem um papel fundamental na regulação da divisão celular, elongação e diferenciação celular (SPAEPEN; VANDERLEYDEN; REMANS, 2007; SULIEMAN, 2011). A técnica de PCR em tempo real permite o monitoramento e a quantificação de transcritos através da análise dos níveis de expressão de genes envolvidos no processo de interação planta-bactéria.

A análise de expressão gênica, realizada através da quantificação dos níveis de transcritos, vem sendo o principal foco de estudos dentro da biologia molecular. Avaliando a quantidade de RNA celular, torna-se possível determinar o quanto que um gene específico está sendo expresso em um organismo em um determinado momento. Para muitos genes, os níveis de expressão podem variar bruscamente de gene para gene, de célula para célula ou em diferentes condições experimentais (SCHMITTGEN; LIVAK, 2008).

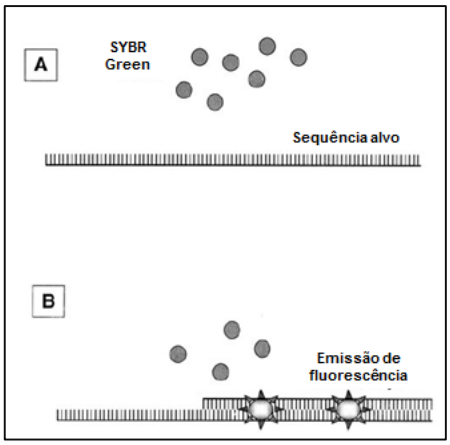
A transcrição reversa combinada com a reação em cadeia da polimerase (RT-PCR) provou ser uma forte ferramenta para quantificar a expressão gênica (LIVAK; SCHMITTGEN, 2001). A introdução de um procedimento baseado na cinética de fluorescência permitiu a quantificação dos produtos de PCR em "tempo real". Assim, a PCR em tempo real é uma técnica mais sensível e acurada do que outros métodos de quantificação de transcritos. Essa técnica é muito mais rápida, pois foi desenvolvida para fornecer

informações tão rapidamente quanto o próprio processo de amplificação, não necessitando manipulações pós-PCR (GIULIETTI et al., 2001).

A PCR em tempo real ou qPCR permite o monitoramento da reação de amplificação em tempo real (ciclo a ciclo), em um sistema fechado, sem interferências externas no progresso da reação. Para isso um sinal fluorescente é detectado em proporção ao aumento da quantidade do produto de amplificação. Esta fluorescência é emitida por compostos que podem estar ligados a sondas, ou compostos que podem ligar-se ou intercalar-se diretamente na dupla fita do DNA amplificado. As sondas (como Taqman®, FRET, Scorpion™) são sequências específicas de DNA que se ligam à região central da sequência-alvo, aumentando a especificidade do método (LIPP et al., 2005; WISEMAN, 2002).

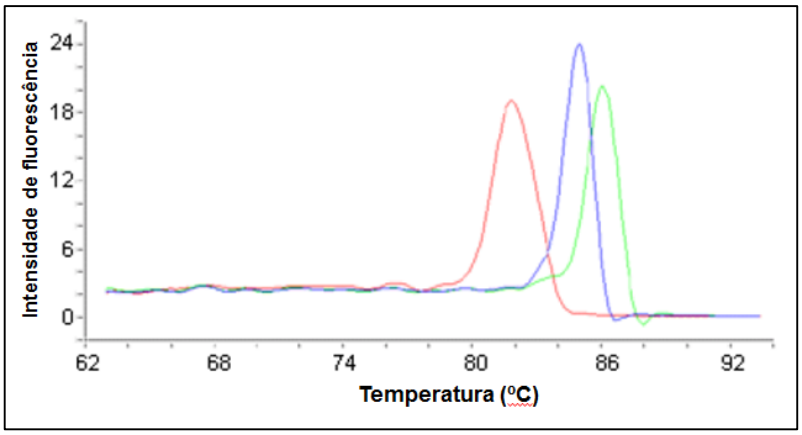
Entre os compostos intercalantes, o mais utilizado é o SYBR Green, o qual se liga inespecificamente à dupla fita de DNA, mas não se liga à fita simples, uma vez que se intercala no sulco menor da dupla fita. Quando este fluoróforo está livre na solução de reação ele não é capaz de emitir fluorescência, a qual será liberada no momento que as moléculas de SYBR Green intercalarem-se nos produtos amplificados. Assim, o aumento da quantidade do DNA sintetizado resulta em um aumento do sinal fluorescente (esquema representativo na figura 1.4). A maior vantagem da utilização desse composto fluorescente é exatamente a não especificidade, uma vez que ele pode ser utilizado com qualquer par de iniciadores para a amplificação de qualquer sequência alvo. Entretanto, esta se torna também a maior limitação desse sistema de quantificação, devido ao risco de detecção de produtos de PCR não específicos, já que o SYBR Green pode ligar-se a qualquer sequência de dupla fita, incluindo dímeros de iniciadores. Contudo, essas amplificações não específicas podem ser diferenciadas pela análise das curvas de dissociação térmica (*melting*), onde diferentes fragmentos geralmente apresentam diferentes temperaturas de dissociação (T_M) e diferentes intensidades (representado na figura 1.5) (GIULIETTI et al., 2001; WEIGHARDT et al., 2004).

Figura 1.4: Representação da amplificação por PCR em tempo real utilizando SYBR Green. (A) SYBR Green livre na solução não emite fluorescência. (B) SYBR Green ligado à dupla fita de DNA, emite fluorescência.



Fonte: Adaptado de Giulietti et al., 2001.

Figura 1.5: Gráfico representando curvas de dissociação térmica (*melting*) de três diferentes produtos de amplificação (linhas de diferentes cores).



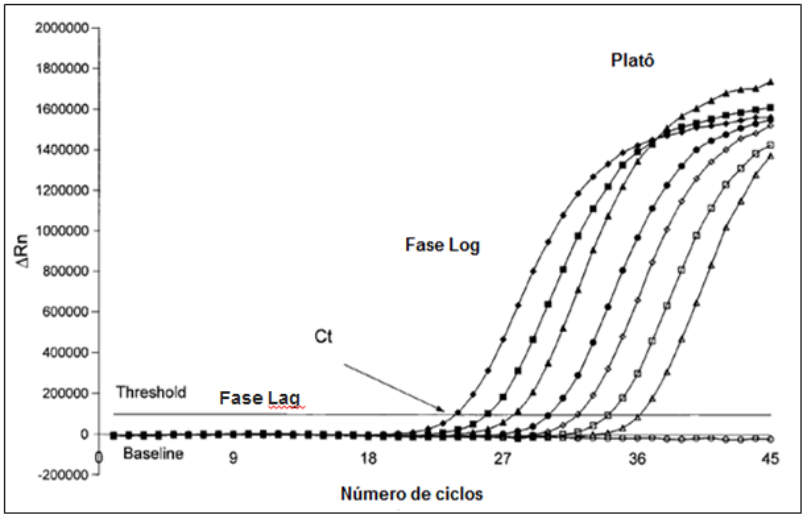
Fonte: Adaptado de Simpson et al., 2000.

A primeira etapa para a realização de quantificação de transcritos por qPCR é a síntese de DNA complementar (cDNA), a qual é catalisada pela enzima transcriptase reversa, utilizando como molde o RNA total extraído de uma amostra. A partir do cDNA sintetizado é realizada a quantificação de transcritos de genes alvos e/ou genes de referência por qPCR (CIKOS; BUKOVSKA; KOPPEL, 2007; LIPP et al., 2005; RUIJTER et al., 2009).

Durante o curso da qPCR ocorrem três fases distintas: a primeira fase é a fase "lag", onde aparece o sinal do ruído de fundo (*background*); a segunda, é a fase exponencial, onde é detectado o sinal fluorescente, uma vez que a quantidade de produtos de amplificação dobra a cada ciclo; a terceira, é a fase final, onde há formação de um platô, sem aumento do número de produtos de amplificação (figura 1.6) (DINON, 2011).

Considerando que um aumento na emissão de fluorescência é detectado na fase exponencial da qPCR, é calculado o ΔRn , cuja equação é $\Delta Rn = Rn^+ - Rn^-$, onde Rn^+ é a emissão de fluorescência do produto a cada ponto (tempo); e Rn^- é a emissão de fluorescência no ponto inicial (*baseline*). Com esses dados, são construídos gráficos de amplificação a cada ciclo, como pode ser visto um exemplo na fig 1.10 (GIULIETTI et al., 2001).

Figura 1.6: Gráfico da amplificação de produtos de PCR em tempo real (ΔRn versus número de ciclos) apresentando as diferentes fases da reação (Lag, exponencial ou Log e platô), bem como o ponto de determinação do C_T.



Fonte: Adaptado de Giulietti et al., 2001.

O ponto de quantificação inicial dos produtos amplificados é o ciclo *treshold* (C_T). Este é definido como o ponto no qual o sinal fluorescente cruza arbitrariamente a linha *treshold*. O limiar de detecção ou linha *threshold* é definido como o nível de sinal fluorescente normalizado, o qual deve estar ajustado acima da linha de base, mas deve ser suficientemente baixo para se encontrar dentro da fase exponencial na curva de amplificação. O valor de C_T detectado é inversamente relacionado com a quantidade de fragmentos amplificados na reação (quanto menor o valor de C_T, maior a quantidade de fragmentos) (SCHMITTGEN; LIVAK, 2008; DINON, 2011). Assim, quanto maior a quantidade inicial da sequência alvo, mais cedo ocorre um aumento significativo da fluorescência e mais baixo será o valor de C_T (WEIGHARDT et al., 2004).

A análise dos dados obtidos por qPCR pode ser feita de duas maneiras: por quantificação absoluta ou quantificação relativa. A quantificação absoluta determina o número de cópias inicial do transcrito de interesse, usualmente relacionando com uma curva padrão interna ou externa. Já a quantificação relativa descreve a mudança na expressão do gene alvo em determinado tratamento em relação a alguma condição de referência, como um

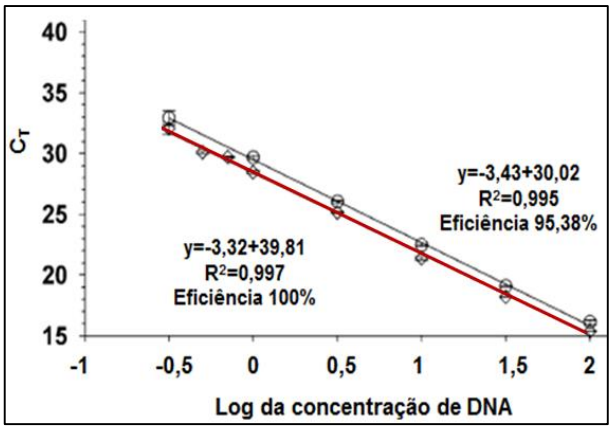
controle não tratado ou uma amostra no tempo zero de tratamento, sendo utilizada, geralmente, para investigar mudanças fisiológicas (LIVAK; SCHMITTGEN, 2001; PFAFFL, 2001; SCHMITTGEN; LIVAK, 2008).

Para análise de expressão gênica por quantificação relativa, é indispensável a seleção de um gene de referência (controle interno ou normalizador), o qual pode ser um gene constitutivo, pois deve ser expresso em nível constante na maioria das células do organismo de estudo, mesmo em estágios de desenvolvimento diferentes ou em órgãos diferentes. Genes envolvidos em processos celulares básicos, como a manutenção da estrutura celular ou do metabolismo primário, são frequentemente escolhidos como normalizadores (ALMEIDA et al., 2010). Após a seleção, esse gene de referência deve ser adequadamente validado para cada experimento para certificar que a expressão gênica não é afetada pelo tratamento ou parâmetros experimentais (LIVAK; SCHMITTGEN, 2001).

A quantificação relativa de transcritos obtidos por qPCR para calcular as variações na expressão gênica pode ser realizada pelo método $2^{-\Delta\Delta CT}$, onde $\Delta\Delta C_T$ é (C_T,alvo – C_T,controle) tempo x – (C_T,alvo – C_T,controle)tempo zero (LIVAK; SCHMITTGEN, 2001). Para o cálculo do $\Delta\Delta C_T$ ser válido, as eficiências (E) de amplificação do gene alvo e do gene de referência devem ser aproximadamente iguais. O valor de E é determinado a partir de uma curva padrão feita pela diluição em série de uma amostra representativa de concentração conhecida (CIKOS et al., 2007). Esta curva padrão é feita tanto para o gene alvo quanto para o gene de referência (controle), e as equações dessas retas vão determinar a similaridade das eficiências (figura 1.7). Dessa forma, se o valor do coeficiente angular (*slope*) das retas for semelhante, as eficiências serão similares e o cálculo do $\Delta\Delta C_T$ pode ser utilizado para quantificação relativa do gene alvo. Uma eficiência de 100% corresponde a duplicação do cDNA a cada ciclo, o que seria ideal, entretanto é aceitável que o valor de eficiência esteja entre 90 e 110%. (LIVAK; SCHMITTGEN, 2001).

Utilizando o método $2^{-\Delta\Delta CT}$, os dados são apresentados em número de vezes que a expressão do gene alvo, normalizado para referência endógena, aumentou ou diminuiu na amostra tratada em relação à amostra controle não tratada (CIKOS et al., 2007).

Figura 1.7: Gráfico representando curvas de validação de um gene de referência (linha vermelha) e um gene alvo (linha cinza) com suas respectivas equações, R^2 e eficiências.



Fonte: o autor

Os dados de expressão relativa podem também ser apresentados como C_T comparativo ou $2^{-\Delta CT}$, onde ΔC_T= (C_T alvo – C_T controle), quando não é considerado diferença entre as amostras (por exemplo, diferentes tempos ou tratamentos das amostras). Geralmente esses dados passam por transformação logarítmica, sendo possível apresentá-los apenas como ΔC_T (LIVAK; SCHMITTGEN, 2001; SCHMITTGEN & LIVAK, 2008). Este cálculo $2^{-\Delta CT}$ é mais adequado quando se tem grupos heterogêneos de amostras, como no caso de plantas individuais, separadas em grupos controle e tratado, e $2^{-\Delta\Delta CT}$ é mais adequado para grupos homogêneos, como cultivo de células (SCHMITTGEN & LIVAK, 2008).

É importante ressaltar que os resultados de quantificação podem variar drasticamente dependendo do método escolhido para a análise dos dados e que, as abordagens analíticas diferentes, podem levar a conclusões opostas (CIKOS et al., 2007). Assim sendo, a escolha da abordagem e do método é de total importância para que os dados obtidos sejam apresentados de forma coerente e realística.

CAPÍTULO 2

ANÁLISE DA EXPRESSÃO GÊNICA EM PLÂNTULAS DE MILHO (VARIEDADE DKB240) INOCULADAS COM A BACTÉRIA PROMOTORA DE CRESCIMENTO VEGETAL *Herbaspirillum seropedicae*

2 ANÁLISE DA EXPRESSÃO GÊNICA EM PLÂNTULAS DE MILHO (VARIEDADE DKB240) INOCULADAS COM A BACTÉRIA PROMOTORA DE CRESCIMENTO VEGETAL *Herbaspirillum seropedicae*

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Resumo

Neste capítulo estão apresentados os resultados obtidos através da análise do nível de expressão de transcritos em plântulas de milho variedade DKB240, inoculadas com a bactéria *Herbaspirillum seropedicae* SmR1. *Herbaspirillum seropedicae* é uma bactéria diazotrófica endofítica, que pode ser uma fonte eficiente de nitrogênio no cultivo de arroz e milho. Neste estudo investigou-se a expressão de genes de raizes de *Zea mays* inoculadas com a bactéria promotora de crescimento vegetal, *Herbaspirillum seropedicae*. Plântulas de milho (cv. DKB240) com três dias de germinação foram inoculadas com *H. seropedicae* cepa SmR1 e cultivadas em areia esterilizada. As coletadas ocorreram aos 1, 4, 7 e 10 dias após a inoculação (DAI), em três repetições biológicas. Analisando parâmetros de crescimento, observamos um número significativamente maior de raízes laterais nas plântulas inoculadas emelacao ao controle aos 7 e 10 DAI. Os níveis de transcrição de 10 genes especificos de milho (*actin1*, *chalcona sintase*, *ent- copalyl difosfato sintase*, *ent- caureno oxidase*, *giberelina 20 oxidase 4*, *auxina1*, *proteína quinase 5*, *proteínas burst respiratorio oxdativo A, B e C*) foram quantificados por qRT -PCR e foi observado um aumento significativo dos níveis de transcrição nos genes *ent- caureno oxidase* e *proteína burst respiratorio oxdativo* C nas amostras inoculadas, em comparação ao controle de 4 DAI. A presença de *Herbaspirillum seropedicae* SmR1 no sistema radicular do milho transitoriamente modula a expressão de um gene envolvido na via de biossíntese de giberelina e de um gene da NADPH -oxidase no início da interação.

GENE EXPRESSION ANALYSIS OF MAIZE SEEDLINGS (DKB240 VARIETY) INOCULATED WITH PLANT GROWTH PROMOTING BACTERIUM *Herbaspirillum seropedicae*

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Abstract

Herbaspirillum seropedicae is an endophytic diazotrophic bacterium, it could be an efficient source of nitrogen in rice and maize cultivation. In this study we investigated the gene expression of *Zea mays* roots inoculated with the plant growth-promoting bacterium *H. seropedicae*. Maize seedlings (cv. DKB240) were inoculated with *H. seropedicae* strain SmR1, grown in sterilized sand and collected 1, 4, 7 and 10 days after inoculation (DAI) in three biological replicates. The number of lateral roots was significantly higher in inoculated seedlings than in control 7 and 10 DAI. Transcript levels of 10 maize genes (*actin1, chalcone synthase, ent-copalyl diphosphate synthase, ent-kaurene oxidase, gibberellin 20 oxidase 4, auxin transporter-like protein 1, mitogen-activated protein kinase 5, respiratory burst oxidase proteins A, B and C*) were quantified by qRT-PCR and a significant increase of transcript levels was observed for *ent-kaurene oxidase* and for *respiratory burst oxidase protein C* in inoculated seedlings compared to control 4 DAI. *Herbaspirillum seropedicae* SmR1 presence in maize roots modulates transiently the expression of one gene involved in gibberellin biosynthesis pathway and one gene of NADPH oxidase in the beginning of interaction.

Keywords *Zea mays*, plant-bacteria interaction, PGPB, qRT-PCR, transcript levels, diazotrophic bacteria

Abbreviations PGPB, plant growth-promoting bacteria; qRT-PCR, quantitative Reverse Transcription Polymerase Chain Reaction; ROS, reactive oxygen species; DAI, days after inoculation.

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2.1 INTRODUCTION

Conventional agriculture is highly dependent on chemical inputs including fertilizers in order to maintain high yields. To keep productivity while reducing chemicals inputs, one strategy is to exploit the potential of plant beneficial microbes as crop inoculants (CANELLAS et al., 2013; LUCY et al., 2004; OLIVEIRA et al., 2009). For cereal crops, prominent plant-beneficial microbes include plant growth-promoting bacteria (PGPB) and arbuscular mycorrhizal fungi (WALKER et al., 2012). PGPB can associate with economically important grasses, such as sugar cane (OLIVEIRA et al. 2009), rice (RODRIGUES et al., 2008; VARGAS et al., 2012), wheat (EL-KOMY et al., 2003) and maize (CANELLAS et al., 2013; RIBAUDO et al., 2001). Beneficial plant-associated bacteria play a key role in supporting and increasing plant health and growth (HUNGRIA et al., 2010; WALKER et al., 2012; COCKING et al., 2003). PGPB can stimulate plant growth by means of several processes, including: biological nitrogen fixation; phosphate solubilization; biological control of pathogens; and the synthesis of hormones such as auxins, cytokinins, gibberellins, ethylene, and a variety of other molecules (HUNGRIA et al., 2010; MONTEIRO et al., 2012). Verhage, Van Wees and Pieterse (2010) studies about colonization of roots by beneficial rhizobacteria suggest that colonization can induces a plant systemic resistance that is effective against a broad spectrum of attackers. The successful PGPB-plant association depends on plant and bacteria genotypes (VARGAS et al., 2012; BALDANI et al., 2000) and it has been demonstrated that the contribution of nitrogen fixation is dependent on the plant variety (JHA et al., 2009). While there have been significant advances in elucidating the details of plant-bacterial interactions in recent years (KARTHIKEYAN et al., 2012; GUTIERREZ-LUNA et al., 2010), many fundamental questions about these processes remain to be resolved, for example, to what extent (primary) plant metabolism is altered by PGPRs and which transcriptional changes are induced by the beneficial interaction (SCHWACHTJE et al., 2011; YANG et al., 2013).

Herbaspirillum seropedicae is an endophytic diazotrophic bacterium (BALDANI et al., 1992; PEDROSA et al., 2011; CHUBATSU et al., 2012), it could be an efficient source of N in rice and maize cultivation (BALDANI et al., 2000; GYANESHWAR et al., 2002). The *Herbaspirillum*-plant association has been studied at the molecular, physiological and microscopical levels, and it has thus become a model to understand the complexities of plant growth-promotion by diazotrophic bacteria (MONTEIRO et al., 2012). *Herbaspirillum*

seropedicae SmR1 is a spontaneous streptomycin resistant mutant of strain Z78 (ATCC 35893) isolated from sorghum (BALDANI et al., 1996), which has had its genome sequenced (PEDROSA et al., 2011).

Based on previous observation that *Herbaspirillum seropedicae* modulates plant defence responses and hormone synthesis during rice colonization (BRUSAMARELLO-SANTOS et al., 2012) and that symbiont bacteria could induce plant immune signaling network (VERHAGE et al., 2010), we decided to evaluate gene expression related to plant defence responses and hormone synthesis during maize colonization. The target genes were chosen by their involvement with plant systemic resistance, hormone synthesis or different stages of plant development and growth regulation.

Auxins are responsible for division, extension, and differentiation of plant cells and tissues. Phytohormones of this group increase the rate of xylem and root formation and also affect photosynthesis, biosynthesis of various metabolites, and resistance to biotic stress factors (BASHAN & DE-BASHAN, 2010). It is well known that some bacteria as *Herbaspirillum seropedicae* also produce auxin. In their interaction with plants, these microorganisms can interfere with plant development by disturbing the auxin balance in plants (SPAEPEN and VANDERLEYDEN, 2011). The Auxin can also act as a signaling molecule in some microorganisms. In general, the main precursor of IAA (Indole 3 Acetamide) biosynthesis is related to tryptophan metabolism (BASHAN & DE-BASHAN, 2010). Auxin transporter-like protein 1 (AUX1) promote lateral root development in plants (MARCHANT et al., 2002; MATSUDA et al., 2011). Genes encoding ent-copalyl diphosphate synthase (*cps1*), ent-kaurene oxidase (*ko1*) and gibberellin 20 oxidase 4 (*ga20ox4*) are involved in the gibberellin synthesis (SONG et al., 2011). Chalcone synthase (*chs*) is involved in the synthesis of defence-related flavonoids (LILLO et al., 2008; LO et al., 2002). Flavonoids are important plant signals for interaction with symbiont bacteria (SANTI et al., 2013) such as flavanone naringenin. This flavonoid was found to regulate genes of *H. seropedicae* predicted to be involved in the colonization process (TADRA-SFEIR et al., 2011), as well as naringenin stimulates the colonization of wheat by *Azospirillum brasilense* and it increases the number of lateral roots cracks in Arabidopsis colonized with *Herbaspirillum seropedicae* (WEBSTER et al., 1998).

The mitogen-activated protein kinase (MAPK) cascade is one of the major pathways by which extracellular stimuli are transduced into intracellular responses in all eukaryotic cells. MAPK pathways play an essential role in signal transduction involved in the regulation of growth, differentiation, proliferation, death and stress responses (SAMAJOVA et al.,

2013). MAPK5 (*mpk5*) is required for the NADPH oxidase-mediated self-propagation of apoplastic H_2O_2 in brassinosteroid-induced antioxidant defence in leaves of maize (ZHANG et al., 2010). Respiratory burst oxidase protein homologues (*rbohA, rbohB* and *rbohC*) are NADPH oxidases involved in generation of reactive oxygen species (ZHANG et al., 2010; ZHANG et al., 2006).

To investigate whether the *H. seropedicae* presence in maize roots could interfere in plant gene expression related to phytohormone metabolism and respiratory burst involved in response to colonizing bacteria, we measured the transcript amounts of ten targets, *actin1 (Zmact), chalcone synthase (Zmchs) , ent-copalyl diphosphate synthase (Zmcps1), ent-kaurene oxidase (Zmko1), gibberellin 20 oxidase 4 (Zmga20ox4), auxin transporter-like protein 1 (Zmaux1), mitogen-activated protein kinase 5 (Zmmpk5), respiratory burst oxidase proteins A, B and C (ZmrbohA, ZmrbohB* and *ZmrbohC),* in *Zea mays* (cv. DKB240) roots inoculated with *Herbaspirillum seropedicae* strain SmR1.

2.2 MATERIAL AND METHODS

2.2.1 Seedling inoculation and growth condition

Seeds of maize (*Zea mays* L. cv. DKB 240) were surface-sterilized using 70% ethanol for 5 min; followed by 1% sodium hypochlorite and 0.01% Tween-20 (USB, Cleveland, OH, USA) solution for 30 min. Seeds were then washed 3 times with sterilized distilled water, transferred to plates containing 0.8% agar-water and maintained for 3 days in growth chamber at 25 ºC, in the dark for germination. *Herbaspirillum seropedicae* strain SmR1 was grown in an orbital shaker (120 rpm) at 30 ºC in 30 mL NFbNHP (NFbN High Phosphate – 1.5 mg/L) medium supplemented with 5 mg/L potassium malate (KLASSEN et al., 1997) until an OD_{600} 0.8 was reached (10^8 cells/mL). Thirty germinated seedlings were incubated in 30 mL of 10^5 cells/mL of *Herbaspirillum seropedicae* SmR1 suspension in NFb malate medium without nitrogen source in an orbital shaker (80 rpm) for 30 min at 30 °C (BALSANELLI et al. 2010). Control seedlings were Mock-inoculated under the same conditions.

Seedlings were washed with 0.9% saline solution and then transferred to sowings containing sterilized sand with Hoagland's solution (HOAGLAND AND ARNON, 1950) without nitrogen. They were maintained in a controlled growth chamber adjusted to 12h

photoperiod, photosynthetic active radiation of 150 µmol m^{-2} s^{-1}, temperature of 25 °C and were watered daily. Hoagland's solution without nitrogen was added over again 7 days after inoculation. Three independent experiments (biological replicates) were performed in different months.

2.2.2 Assessment of growth parameters

Maize seedlings were randomly collected 1, 4, 7 and 10 days after inoculation (DAI). Root and shoot fresh weight and length as well as number of lateral roots were measured. Material was immediately frozen at liquid nitrogen and stored at -80°C for RNA extraction. Weight and length data were analyzed by nonparametric *Kruskal-Wallis* test (P <0.05). Number of lateral roots was analyzed by ANOVA and compared by F-test. Means were compared using the post-hoc test of Tukey's multiple range (P <0.05). Data were analyzed using the package Agricolae on R software v2.15.3 (R Development Core Team. R: A language and environment for statistical computing. http://www.R-project.org/).

2.2.3 Root colonization assay

To determine internal bacterial colonization, three seedlings from each treatment were randomly collected 1, 4, 7 and 10 DAI. Roots were surface-sterilized with 70% ethanol for 2 min, followed by 1% sodium hypochlorite for 2 min and washed 3 times in distilled water. Root material from each individual plant was weighed and macerated using a sterile pestle and mortar in sterile saline solution (0.9% NaCl) in proportion 1:10. Homogenates were ten times serially diluted and 10 µL from each homogenate were dispensed on the agar plate with NFb malate medium containing streptomycin (80 µg/mL) with a fixed number of separated small drops by drop plate method (HERIGSTAD et al., 2001). After 2 - 3 days of incubation the colonies were determined as most probable number (MPN) per gram of root fresh weight (PEDRAZA et al., 2009; OLIVEIRA et al., 2009).

2.2.4 RNA isolation and cDNA synthesis

Total RNA isolation and cDNA synthesis were carried out as described previously (Hermes et al. 2013) from approximately 100 mg of root material. Nucleic acids were quantified by the Nanodrop ND 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). For the RNA isolation, three seedlings of each time and of each biological replicate were used, thereby nine isolated RNA were prepared for each condition.

2.2.5 Expression analysis by qRT- PCR

The primer sequences and concentrations used for quantitative reverse transcription PCR (qRT-PCR) are shown in Table 1. To determine amplification efficiencies, cDNA of a control sample was serially diluted in water (5-fold dilution from 100 ng to 0.16 ng of cDNA template) and relative standard curves for each target gene were compared to the reference gene (α-tubulin) in triplicate reactions. Amplification efficiencies were determined using the followed equation: Efficiency = $10^{(-1/slope)}$-1, where slope is the value obtained from the standard curve. The sample reactions were performed using 100, 300 or 500 nM of primers (Table 2.1), 10 μL SYBR Green PCR Master Mix 2X (Life Technologies, Carlsbad, CA, USA) and 40 ng of template cDNA. Amplification reactions of all samples of the same biological replicate were carried out in triplicate in adjacent wells on the same plate on an ABI PRISM^{TM} 7500 detection system (Applied Biosystems, Foster City, CA, USA) as described previously (Hermes et al. 2013; Mello et al. 2012). The transcript relative quantification of target genes was performed in relation to α-tubulin (reference gene) and it was calculated by $\Delta C_t = Ct_{target} - Ct_{reference}$ and values were expressed as $2^{-\Delta CT}$ (SCHMITTGEN AND LIVAK, 2008). The control samples (Mock-inoculated) 1 DAI were used as normalized values, one-fold transcript levels. Fold change in transcript levels were analyzed by nonparametric *Kruskal-Wallis* test and compared by *t-test* (P <0.05) between inoculated and control roots for each harvest time**.** Statistical analysis was performed using R software v2.15.3.

Table 2.1: Primer sequence used in qRT-PCR analysis for *Zea mays* transcript quantification.

***Zea mays* gene**	**Concentration (nM)**	**Primer sequence**	**GenBank Accession No.**	**Reference**
α-tubulin	300	GCGCACCATCCAGTTCGT	X73980	Mello, 2012
	300	CTGGTAGTTGATTCCGCACTTG		
β-actin	300	GCAGCATGAAGGTTAAAGTGATTG	NM_001155179.1	Mello, 2012
	300	GCCACCGATCCAGACACTGT		
chalcone synthase	300	CGTCCGTCCGCAAATAATGT	NM_001148774.1	Mello, 2012
	300	ATGATGATTGTGCGACTGACAGT		
ent-copalyl diphosphate	300	ATGATGAGCCATGTCGATGA	AY562491	Song *et al.;* 2011
synthase	300	GAAAGGTCTGCCTTGTCTCG		
ent-kaurene oxidase	300	CAATCTGTACGGGTGCAACA	BT042205	Song *et al.*; 2011
	300	CCTCTAGGTGCGAGGTACACAT		
gibberellin 20 oxidase 4	500	GAGAGGTTCTCCATGCCCTA	NM_001156071	Song *et al.*; 2011
	100	AAGAAGTCGCCCCAGTTGTA		
auxin transporter-like	300	CGAAACGCACCCTGCATT	NM_001158094.1	This study
protein 1	500	CCCGCTTTTTACAGTGGAAGAT		
MAP kinase 5	500	TCTGCTCGGCGGTCAACT	AB016802	Zhang *et al.*; 2010
	100	AAGGCGTTGGCGATCTTCTT		
respiratory burst oxidase	100	CACACGTGACCTGCGACTTC	DQ855284	Zhang *et al.*; 2010
protein A	300	CCCCAAGGTGGCCATGA		
respiratory burst oxidase	500	GGCCAGTACTTCGGTGAAACA	EU807966	Zhang *et al.*; 2010
protein B	300	ATTACACCAGTGATGCCTTCCA		
respiratory burst oxidase	100	TTCTCTTGCCTGTATGCCGC	DQ897930	Zhang *et al.*; 2010
protein C	500	CTTTCGTATTCCGCAGCCA		

2.3 RESULTS

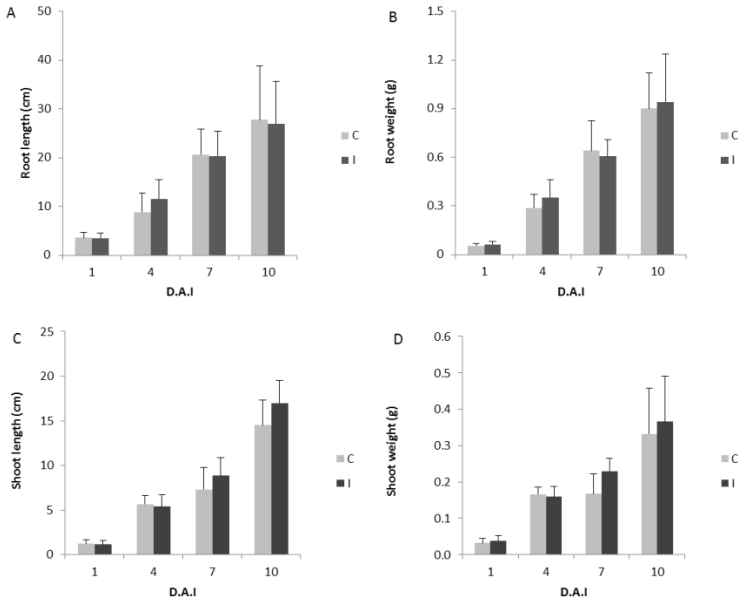
Maize seedlings (cv. DKB 240) were inoculated with 10^5 cells/seed of *H. seropedicae* strain SmR1, grown in sterilized sand and harvested 1, 4, 7 and 10 DAI. The colonization of maize roots by *H. seropedicae* SmR1was evaluated by most probable number (MPN). The root colonization assay showed that 10^5 MPN/g was presented in inoculated roots 1, 4 and 7 DAI, while 10^4 MPN/g was presented in inoculated roots 10 DAI (Table 2.2). The bacteria able to grow in NFbHP malate medium containing 80 µg/mL streptomycin was not detected in control roots.

Table 2.2: Number of bacteria (log_{10} MPN/g fresh root) of maize seedlings (DKB240 variety) grown in sand after inoculation with *Herbaspirillum seropedicae* strain SmR1. Roots were collected 1, 4, 7 and 10 days after inoculation (D.A.I.). Three independent experiments produced similar results.

D.A.I.	log_{10} MPN/g
1	5
4	5
7	5
10	4

The growth parameters (length and fresh weight of root and shoot) were measured 1, 4, 7 and 10 DAI and differences were not observed between inoculated and control seedlings (Figure 2.1). Otherwise the number of lateral roots was significantly higher in inoculated seedlings than in control 7 and 10 DAI (Figure 2.2).

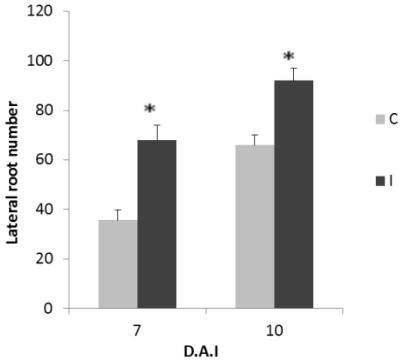
Figure 2.1. Growth parameters of maize seedlings (DKB240 variety) grown in sand after inoculation with *Herbaspirillum seropedicae* strain SmR1. Control (C) and inoculated (I) samples collected 1, 4, 7 and 10 days after inoculation (D.A.I). (A) Root length, (B) Root fresh weight, (C) Shoot length and (D) Shoot fresh weight. *Kruskal-wallis* test followed *t-test* ($P<0.05$) was performed and no significative difference was observed between control and inoculated samples. Data are presented as means ± SD (n=27).



In order to obtain information about the early events involved in maize-*H. seropedicae* SmR1 interaction, we investigated the expression profile of ten maize genes (*Zmact, Zmchs, Zmcps1, Zmko1, Zmga20ox4, Zmaux1, Zmmpk5, ZmrbohA, ZmrbohB* and *ZmrbohC*) of inoculated and control roots. The transcript levels were quantified by qRT-PCR (ΔC_T method) in maize roots 1, 4, 7 and 10 DAI using α-tubulin as internal control gene. First we determine the amplification efficiency of the target genes and internal control gene (Schmittgen and

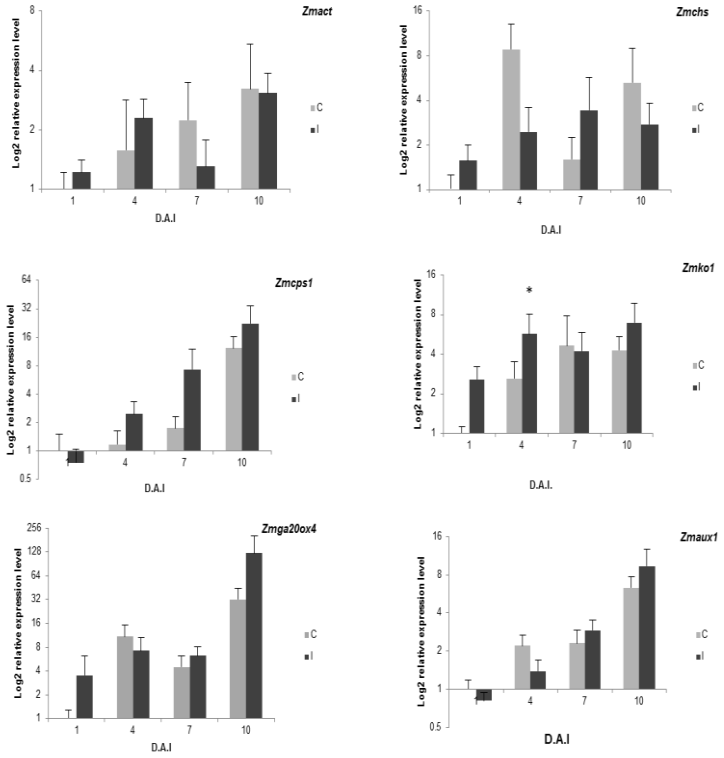
Livak, 2008). The amplification efficiencies (E) for the analyzed genes were in the range 90 - 110% values for ΔC_T analysis for 8 targets, whilst E were 75% and 86% for *Zmga20ox4* and *Zmcps1*, respectively (Table S1).

Figure 2.2:. Number of lateral roots of maize seedlings (DKB240 variety) grown in sand after inoculation with *Herbaspirillum seropedicae* strain SmR1. Control (C) and inoculated (I) samples collected 7 and 10 days after inoculation (D.A.I). Statistical analysis was performed using the *Tukey test* (P<0.05). Asterisk (*) indicates a significant difference. Data are presented as means ± SD (n = 3).



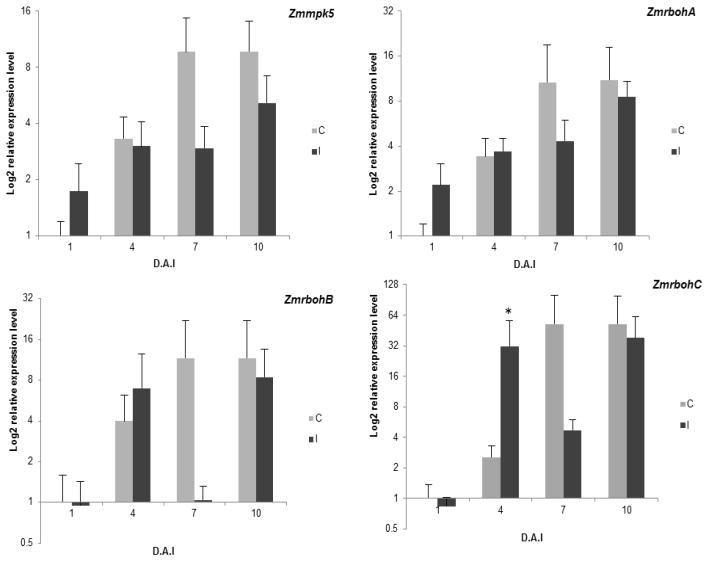
The internal control gene α-tubulin presented constant transcript amounts in all analyzed sample groups. At distinct time points, 1, 4, 7 and 10 DAI, the transcript levels of actin1 (*Zmact*) and chalcone synthase (*Zmchs*) presented similar transcript amounts in control and inoculated samples (Figure 2.3). The transcripts levels of ent-copalyl diphosphate synthase (*Zmcps1*) presented an increase 10 DAI comparing to 1 DAI, for both control and inoculated samples. For ent-kaurene oxidase 1 (*Zmko1*) transcript levels, significant increase was observed 4 DAI in inoculated maize roots comparing to control values (Figure 2.3). The transcript levels of gibberellin 20 oxidase 4 (*Zmga20ox4*) presented an increase 10 DAI comparing to 1 DAI, in both control and inoculated samples. For auxin transporter-like protein 1 (*Zmaux1*) transcripts, the values were similar in control and inoculated samples (Figure 2.3).

Figure 2.3: Relative expression level of *act, chs, cps1, ko1, ga20ox4* and *aux1* in roots of maize seedlings (DKB240 variety) grown in sand after inoculation with *Herbaspirillum seropedicae* strain SmR1. Control (C) and inoculated (I) samples collected 1, 4, 7 and 10 days after inoculation (D.A.I). Relative amounts of transcripts were calculated by qRT-PCR using ΔC_T method and α-tubulin as reference gene. *Kruskal-wallis* test followed *t-test* ($P<0.05$) was performed and asterisk (*) indicates a significant difference. Data are presented as means ± SE (n = 9).



For mitogen-activated protein kinase 5 (*Zmmpk5*), respiratory burst oxidase proteins A and B (*ZmrbohA* and *ZmrbohB*) transcripts, the relative expression values were similar in control and inoculated samples (Figure 2.4). For respiratory burst oxidase protein C (*ZmrbohC*), transcript levels presented significant increase in inoculated maize roots 4 DAI comparing to control roots 4 DAI (Figure 2.4).

Figure 2.4: Relative expression level of *mpk5*, *rbohA*, *rbohB* and *rbohC* in roots of maize seedlings (DKB240 variety) grown in sand after inoculation with *Herbaspirillum seropedicae* strain SmR1. Control (C) and inoculated (I) samples collected 1, 4, 7 and 10 days after inoculation (D.A.I). Relative amounts of transcripts were calculated by qRT-PCR using ΔC_T method and α-tubulin as reference gene. *Kruskal-wallis* test followed *t-test* ($P<0.05$) was performed and asterisk (*) indicates a significant difference. Data are presented as means ± SE ($n = 9$).



2.4 DISCUSSION

It is well known that roots are the primary site of interaction between plants and microorganisms, playing an important role in this process (MERCADO-BLANCO AND PRIETO, 2012). Here we demonstrated maize root colonization by *H. seropedicae* SmR1 that was analyzed 1, 4, 7 and 10 DAI in order to monitor the growth of bacteria during the different days. The number of bacteria per g of root (fresh weight) was around 10^4-10^5 MPN/g in all harvest times (Table 2). Internal colonization by *H. seropedicae* SmR1 was previously demonstrated in roots and shoots of maize 10 DAI, the bacterial population was around 10^6 and 10^4 CFU/g of dry weight, respectively (RONCATO-MACCARI et al., 2003). Using bacteria labeled with red fluorescent protein, it was observed that 1 DAI the root, including the xylem, is completely colonized by *H. seropedicae* SmR1. Its attachment to maize roots was observed 30 min after inoculation and root invasion by *H. seropedicae* SmR1 was a very rapid process, and it occurred in the absence of any visible defence response by the plant (MONTEIRO et al., 2008) therefore without damaging the host or eliciting symptoms of plant disease (MONTEIRO et al., 2008; REINHOLD-HUREK AND HUREK, 2011) that could

display a mechanisms of action to protect host. It has been known that associative beneficial bacteria can increase their host growth, however in particular cases despite the optimistic expectations on the impact of associative diazotrophic nitrogen fixation, most inoculation experiments did not show substantial contribution to plant growth (DOBBELAERE et al., 2003). It may explain the absence of significance difference in the growth parameters analyzed in root and shoot length as well as fresh weight between control inoculated plants in the beginning of interaction in our work. Also the bacterial and plant genotypes can interfere in the association and growth promotion (VARGAS et al., 2012). However, there are several ways that PGPB can enhance and support plant growth and development in plants. Among them, production of phytohormones such as auxins, cytokinins, gibberellins, ethylene, and a variety of other molecules (HUNGRIA et al., 2010; MONTEIRO et al., 2012). The phythormones produced by bacteria thus enhance root branching and root elongation, which in turn favour the uptake of soil water and also minerals resulting a positive effect on plant growth (STEENHOUDT AND VANDERLEYDEN, 2000). As early effect of inoculation, we have observed significant increase of the number of lateral roots in inoculated maize seedlings comparing to control 7 and 10 DAI (Figure 2.2). Similar to our results a significant increase in lateral root numbers were observed in two rice varieties 10 DAI with PGPB *A. brasilense* (VARGAS et al., 2012) and in tomato plants inoculated with *Herbaspirillum seropedicae* (BOTTA et al., 2013). The early effects on maize secondary metabolism were investigated 16 DAI with PGPB *Azospirillum lipoferum* strain CTR1 and *Pseudomas fluorescens* strain F113 and inoculation did not impact on plant biomass or in root length but resulted in enhanced total root surface, total root volume and root number (WALKER et al., 2012).

Upon inoculation of plants with PGPR, a change in root architecture is observed, mainly as an increase in root hairs and lateral roots and shortening of the root length. The plant growth promoting effect of bacteria, for which it has been shown that auxin is involved, is known as phytostimulation (SPAEPEN AND VANDERLEYDEN, 2011). The mechanisms behind PGPR/plant interaction are poorly understood, as most studies have described short-term responses on plants and only a few studies have analyzed plant molecular responses under PGPR colonization (POUPIN et al., 2013). Concerning plant gene expression response to *H. seropedicae*, SmR1, we observed significant increased amounts 4 DAI of *ent-kaurene oxidase (Zmko1)* (Figure 2.3) and *respiratory burst oxidase homologue protein C* (*ZmrbohC*) transcripts in inoculated maize seedlings comparing to control seedlings (Figure 2.4). *Zmko1* is involved in the second stage of gibberellin biosynthesis pathway (SONG et al., 2011). Gibberellin is an essential phytohormone that controls many aspects of plant development

(SONG et al., 2011) and seems to be involved in the plant immune signaling network with salicylic acid, jasmonates and ethylene (VERHAGE et al., 2010). *ZmrbohC* is a NADPH oxidase involved in generation of reactive oxygen species (ROS), participating in plant defence response, cell death, abiotic stress, hormone signalling, stomatal closure, and root hair development (LIN et al., 2009). NADPH oxidase complex is considered one of the most important sources of superoxide anion in the plant cell (GROPPA et al. 2012). In this way, our results hypothesized that the increase on transcript level of *Zmko1* and *ZmrbohC* at 4 DAI in inoculated samples could be related to the early signaling plant defence response to the bacterial colonization. It is possible that at 7 DAI the bacterial colonization have been already established. Regarding the results of 10 DAI all of genes showed similar behavior to control plants, it means that the bacteria presence may not affect the plant system after establishing the association. A study with *Arabidopsis* inoculated with *Burkholderia phytofirmans* strain PsJN was analyzed 14 DAI and it showed an up regulation of gene *AtGA3ox1*. This gene is involved on gibberellin pathway. The enzyme catalyzes the final step in the synthesis of bioactive gibberellin (POUPIN et al., 2013). The gibberellin produced by *Azospirillum* was found to play an important role in the early stages of plant growth in Gramineae by enhancing shoot and root growth and also increasing root hair density (SANTI et al., 2013).

A high number and diversity of genes encoding enzymes potentially involved in the detoxification of ROS were found investigating the rice endophyte metagenome (SESSITSCH et al., 2012). Plants produce a range of ROS in response to abiotic stress or to colonizing microorganisms which elicit an oxidative burst, thus the abundance in the metagenome suggests that endophytes require these enzymes to be able to successfully colonize plants (SESSITSCH et al. 2012).

In accordance with our results, other studies showed modulation of gramineous defence response genes by PGPB in early stages of interaction. In rice roots inoculated with *H. seropedicae* SmR1, the expression of stress- and defence-related genes was decreased while expression of metallothionein gene was increased (BRUSAMARELLO-SANTOS et al. 2012). In rice roots inoculated with *Azospirillum brasilense*, the expression of ethylene receptors was increased 3 DAI (VARGAS et al. 2012). In maize roots inoculated with *Azospirillum lipoferum* strain CTR1, benzoxazinoids contents were changed (WALKER et al. 2012). Benzoxanizoids are defence molecules of great importance in biotic interactions, since they inhibit *vir* genes of *Agrobacterium tumefaciens* (ZHANG et al. 2000) among other effects. In our previous work using maize roots inoculated with *A. brasilense*, we observed a significant decrease of a protein presenting homology to hydroxyproline-rich glycoprotein-

like protein (CANGAHUALA-INOCENTE et al., 2013), that is involved in defence signaling pathway (SUJEETH et al., 2012).

In this work, we analyzed the expression of ten genes chosen by their involvement in plant systemic resistance and hormone synthesis. In the future, we envisage to analyze the global response of maize-*H. seropedicae* SmR1 interaction using RNA sequencing. Recently maize transcriptome based on RNA sequencing was published (SEKHON et al., 2013) and it is available on Maize Genetics and Genomics Database (www.maizeGDB.org).

2.5 CONCLUSION

We observed that *Herbaspirillum seropedicae* SmR1 presence in maize roots increased the expression of one gene involved in gibberellin biosynthesis pathway (*Zmko1*) and one gene of NADPH oxidase (*ZmrbohC*) 4 DAI. It suggests that gibberellin synthesis and respiratory burst oxidase pathways could be regulated by this endophytic bacterium presence in maize roots in the beginning of interaction. This work support the hypothesis previously proposed that *Herbaspirillum seropedicae* modulates plant defence responses and hormone synthesis during graminaceous colonization (BRUSAMARELLO-SANTOS et al., 2012).

Understanding the bacterial gene expression analysis could be interesting to figure out the mechanisms and pathways used by beneficial bacteria to promote plant growth. This information could be exploited to knowledge about dynamics and establishment of interaction between PGPB and crops plants.

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CAPITULO 3

O USO DE CARBONO-11 PARA MEDIR RESPOSTAS FISIOLÓGICAS E METABÓLICAS DE PLANTAS DE *Setaria viridis* INOCULADAS COM *Azospirillum brasilense e Herbaspirillum seropedicae*

O USO DE CARBONO-11 PARA MEDIR RESPOSTAS FISIOLÓGICAS E METABÓLICAS DE PLANTAS DE *Setaria viridis* INOCULADAS COM *Azospirillum brasilense* e *Herbaspirillum seropedicae*

Fernanda Plucani do Amaral[1,5], Beverly Agtuca[2], Vania Carla Pankievicz[3], Ana Carolina Maisonnave Arisi[1],Emanuel M de Souza[3] Michael J. Schueller[5], Gary Stacey[6] e Richard A. Ferrieri[5]

Resumo

Nós apresentamos um estudo detalhado que aborda as bases fisiológicas e metabólicas para entender associações entre planta-rizobactérias promotora de crescimento vegetal (PGPR) no contexto da capacidade de sustentar o crescimento saudável da planta hospedeira sob limitação de nitrato. Neste estudo utilizamos $^{11}CO_2$ radioativo (t ½ 20,4 min) administrado em *Setaria viridis*, uma gramínea modelo C_4, para quantificar vários atributos básicos da fisiologia vegetal envolvidos no crescimento da planta, incluindo: i) a entrada de carbono via fixação de $^{11}CO_2$, (ii) o transporte de ^{11}C - fotoassimilados da folha, (iii) alocação de ^{11}C – fotoassimilados para as raízes, e (iv) exsudação desses recursos para a rizosfera. Em conjunto, as informações destes quatro atributos forneceram uma visão abrangente de como as plantas usam seus recursos de carbono quando submetidas a três diferentes condições de crescimento: (i) plantas de *S. viridis* cultivadas em condições normais de nitrato (5mM NO_3^-); (ii) *S. viridis* cultivadas sob limitação de nitrato (0,5mM de NO_3^-), e (iii) *S. viridis* inoculadas com bactérias e cultivadas sob limitação de nitrato (0,5mM de NO_3^-). Além disso, submetemos o tecido foliar radioativo a análises radiometabólicas que forneceram informações sobre a assimilação do carbono, administrado como ^{11}C, em componentes solúveis como *pool* de açúcares e aminoácidos bem como componentes insolúveis. Nossos resultados mostraram uma diminuição na fixação de $^{11}CO_2$ nas plantas não inoculadas submetidas a limitação de nitrato em relação aos controles, porem houve aumento das exportações de ^{11}C - fotoassimilados para as raízes. A exsudação radicular também foi significativamente reduzida nas plantas inoculadas. Além disso, a reprogramação metabólica do novo carbono assimilado em aminoácidos à custa de açúcares solúveis também foi proeminente. No entanto, plantas inoculadas com PGPR submetidas à mesma limitação de nitrato mostrou um retorno a estados fisiológicos e metabólicos de plantas controle cultivadas em condições normais de nitrato, apresentando algumas exceções como uma porcentagem maior de açúcares ^{11}C - monossacarídeos relativos aos controles, e também um percentual maior de ^{11}C - glutamina, devido à diminuição do metabolismo deste aminoácido.

3 CARBON-11 USED TO MEASURE THE PHYSIOLOGICAL AND METABOLIC RESPONSES OF *Setaria viridis* INOCULATED WITH *Azospirillum brasilense* AND *Herbaspirillum seropedicae*

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Abstract

We present a detailed mechanistic study that addresses the physiological and metabolic basis for understanding PGPR associations in the context of host ability to sustain healthy growth under nitrate limitation. Our studies used radioactive $^{11}CO_2$ ($t_{½}$ 20.4 min) administered to *Setaria viridis*, a model C_4 grass, to quantify several basic attributes of plant physiology that shape plant growth including: i) carbon input via $^{11}CO_2$ fixation; (ii) leaf export of ^{11}C-photoassimilates; (iii) allocation of ^{11}C-photoassimilates belowground to roots; and (iv) exudation of these resources to the surrounding rhizosphere. Taken together, information from these four attributes was leveraged to provide a comprehensive look at how plants use their carbon resources when subjected to three different environmental growth regimes encompassing: (i) *S. viridis* grown under normal nitrate conditions (5mM NO_3^-); (ii) *S. viridis* grown under nitrate-limitation (0.5mM NO_3^-); and (iii) bacterial inoculated *S. viridis* grown under nitrate-limitation (0.5mM NO_3^-). Additionally, we subjected source leaf tissues to radiometabolite analyses giving insight into the chemical partitioning of new carbon (as ^{11}C) into the soluble sugar and amino acid pools, and the insoluble storage components. Our findings showed that uninoculated plants subjected to nitrate limitation had decreased $^{11}CO_2$ fixation relative to controls, but increased export of available ^{11}C-photoassimilates belowground to roots. Root exudation was also significantly reduced. Furthermore, metabolic reprogramming of new carbon into amino acids at the expense of soluble sugars was also prominent. However, PGPR inoculated plants subjected to the same nitrate limitation showed a return to normal physiological and metabolic states of unstressed control plants with a few exceptions: they had a higher percentage of ^{11}C-monosaccharidic sugars relative to controls,

and they had a higher percentage of ^{11}C-glutamine owing to decreased metabolism of this amino acid.

Keywords Carbon-11. *Setaria viridis. Azospirillum brasilense*. *Herbaspirillum seropedicae.* Plant-bacteria interaction.

3.1 INTRODUCTION

Nitrogen (N) is the major limiting nutrient that promotes growth of most plants. Acquisition and assimilation of N is second in importance only to photosynthesis for plant growth and development (SULIEMAN, 2011). For this reason common agricultural practices make use of chemical inputs including fertilizers to maintain high crop yields. Excessive use of fertilizers, however, can have the adverse effects on the environment through extensive chemical runoff into the waterways (WALKER et al., 2012). Agricultural practices are mindful of this and as a result are attempting to implement more environmentally friendly ways to address crop sustainability (BOTTA et al., 2013). For example, plant beneficial microbes have been exploited as inoculants (LUCY et al., 2004; OKON, Y.; LABANDERAGONZALEZ, 1994) to enhance plant growth and crop productivity while minimizing field deployment of chemicals.

Plant growth promoting bacteria (PGPB) are suspected to enhance plant growth for a number of reasons. Among them, these bacteria are diazotrophs, and therefore are capable of fixing atmospheric N_2 potentially providing a source of biological nitrogen to their host (FRANCHE et al., 2009; LAM et al., 1996). These bacteria, however, are also capable of producing various phytohormones that can improve root growth, and in turn, enhance water and mineral nutrient uptake for increased plant growth and plant fitness enabling them to better withstand environmental challenges such as drought, predation by herbivores or attack by pathogens (HUNGRIA, M. et al., 2010; MONTEIRO et al., 2012).

Members of the Poaceae family have long been known to positively associate with particular species of PGPB including those in the genera *Azospirillum*, *Burkholderia*, *Gluconacetobacter* and *Herbaspirillum* (DOBEREINER, 1992; JAMES, E. K.; OLIVARES,F.L.,1998; SUAREZ-MORENO et al., 2012). *Azospirillum brasilense*, in particular, is a rhizobacteria that associates well with plant roots via formation of biofilms on the outer surface. *Herbaspirillum seropedicae*, on the other hand, is an endophytic bacteria that associates with plant roots by colonizing the xylem vascular tissues within the roots (SANTI et al., 2013). Both colonize their host often in high numbers, without damage and without eliciting symptoms of plant disease (REINHOLD-HUREK; HUREK, 2011).

Both of these bacteria are able to establish associations with grasses of agronomic importance including maize (*Zea mays*), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*) and sugarcane (*Saccharum* sp) (JAMES, E. K.; OLIVARES, F. L., 1998; VARGAS et al., 2012).

In general, C_4 grasses have exhibited gains in C_4 photosynthesis which may be inherently due to improved nitrogen uptake and nitrogen use efficiency. These actions can help elevate overall crop productivity in several major food crops and bioenergy grasses (BRUTNELL et al., 2010).

Setaria is a diploid C_4 grass with small genome of 515 Mb. This species of grass is closely related to the major food and feed crops maize and sorghum (LI, P.; BRUTNELL, 2011) which makes it an attractive model system for studying C4 grass associations with microorganisms. *Setaria viridis,* the weedy relative of *Setaria italica,* also possesses attributes suitable for genetic analyses including a small stature, rapid life cycle, and prolific seed production. *Setaria* sp. is also morphologically similar to most Panicoideae grasses, including potential biofuel feedstocks including switchgrass (*Panicum virgatum*) and Miscanthus (*Miscanthus giganteus*) (BRUTNELL et al., 2010). Therefore, understanding the mechanisms underpinning growth promotion by bacteria associations in this model grass system could have far reaching benefits to both agriculture and energy.

The goal of the present work was to lay the groundwork for quantifying the physiological and metabolic responses of *Setaria viridis* to several growth regimes including normal N-fertilization, low N-fertilization, and low N-fertilization in the presence of both *A. brasilense* and *H. seropedicae* PGPR bacteria. Here we used the short-lived radioactive isotope, carbon-11 ($t_{1/2}$ 20.4 min), administered to intact leaves as $^{11}CO_2$ for rapid assimilation into photoassimilates comprised largely of mobile sugars and amino acids. Carbon-11 (^{11}C) has been successfully used to measure the spatial and temporal profiles defining the movement not only of ^{11}C-photoassimilates in plants (AGTUCA et al., 2013; FERRIERI et al., 2013), but also the movement of other substrates including ^{11}C-phytohormones (THORPE et al., 2007).

3.2 MATERIALS AND METHODS

3.2.1 Plant Growth and Treatments

Seeds of *Setaria viridis* genotype A10.1 were surface-sterilized for 3 min in a solution comprised of 1 mL of bleach (6.15% of sodium hypochlorite) and 1 µL of Tween 20. Seeds were then consecutively washed 5 times using deionized water. These sterilized seeds were

planted in 100 mL glass test tubes (Fisher Scientific, Inc., Pittsburg, PA, USA) filled with Hoagland's fortified agar gels adjusted to pH 5.9. For the uninoculated control studies, the nitrate level was adjusted to either 5.0 mM nitrate (here called normal nitrate level) or 0.5 mM nitrate (limiting nitrate level). For the bacterial inoculated studies, the nitrate level in the gel was adjusted to 0.5 mM nitrate.

The Hoagland's solutions were prepared from the individual salts in order to control the nitrate concentrations listed above. Gel solutions were prepared using 2 L of distilled water, 1 mL of 1 M potassium phosphate monobasic, 1 mL of 1 M potassium dibasic, 1 mL of 1 M micronutrient solution, 1 mL of 0.028 M ferric ethylenediaminetetraacetic acid, 2 mL of 1 M calcium chloride, 2 mL of 1 M magnesium sulfate, potassium nitrate (adjusted to obtain either a 5.0 mM or 0.5 mM nitrate concentration), 1.1 g MES hydrate, and 5.6 g Gelzan CM. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO USA). Solutions were autoclaved (Harvey SterileMax, Thermo Fisher Scientific, Inc., Pittsburg, PA, USA) for 15 min at 121°C and mixed at high speed to enable aeration of the viscous solution before it had a chance to set as a gel. All growth tubes were placed and cultivated in a commercial growth chamber (Percival, Inc) at 23°C using 120 µmol m^{-2} s^{-1} of light intensity on a 12 h photoperiod. These three-week-old plants had a height of about 5 cm and were used for experiments.

3.2.2 Bacterial Growth and Seeds Inoculation

H. seropedicae was grown in 10 mL NFbHPN (HP means high phosphate) medium with malate 20% (KLASSEN et al., 1997), while *A. brasilense* was grown in 10 mL NFbHPN medium with lactate 50% (PEDROSA; YATES, 1984), at 120 rpm, 30°C in orbital shaker (Max Q 4450; Thermo Fisher Scientific, Inc., Pittsburg, PA, USA). The surface-sterilized seeds were inoculated with washed bacterial cultures ($O.D_{600}$ =1; 10^7 cells mL^{-1}) diluted in sterile, nitrogen depleted Hoagland solution. The seeds were inoculated by absorption of 50 µL of bacterial mixture for about 40 min. The bacterial culture was made with a combination of *H. seropedicae* strain Ram4 and *A. brasilense* strain FP2-7.

3.2.3 Root Colonization Assay

All materials for this process were sterilized prior to use. To determine total and internal bacteria colonization, three seedlings from each treatment were collected 15 days after inoculation and their roots were surface-sterilized separately. For a total bacterial count assay, roots were washed 3 times using distilled water. For an internal colonization assay, root tissues were washed for 2 min using a 70% ethanol solution containing sodium hypochlorite (6% v/v) then washed 3 times using distilled water. All root tissues were weighed and ground in saline solution (0.9% sodium chloride) using a mortar and pestle. Ten-fold serial dilutions were from these extracts and each dilution was plated onto NFb malate medium for *H. seropedicae* and NFb lactate medium for *A. brasilense*. For determining the bacterial count, the drop plate method was performed and the number of cells was analyzed after 3 days of incubation at 30°C.

3.2.4 Radioisotope Production and Radiotracer Administration

$^{11}CO_2$ was produced *via* the $^{14}N(p, \alpha)^{11}C$ nuclear transformation (Ferrieri & Wolf, 1983) from a 20 mL target filled with high-purity nitrogen gas (400 mL @ STP) using 18MeV protons from the TR-19 (Ebco Industries Ltd, Richmond, BC, Canada) cyclotron at BNL, and captured on a molecular sieve (4Å). The $^{11}CO_2$ that was trapped on the molecular sieve was desorbed and quickly released into an air stream at 200 mL/min as a discrete pulse for labeling a leaf affixed within a 5 x 10 cm lighted (320 µmol m^{-2} s^{-1}) leaf cell at 21ºC to ensure a steady level of fixation. The load leaf affixed within the cell was pulse-fed $^{11}CO_2$ for 1 minute, then chased with normal air for the duration of exposure. A PIN diode radiation detector (Carroll Ramsey Associates, Inc, Berkeley, CA, USA) affixed to the bottom of the leaf cell enabled continuous measurement of radioactivity levels within the cell during the initial pulse and in the minutes right after the pulse giving information on $^{11}CO_2$ fixation.

3.2.5 Plant Physiological Measurements

Four components of plant physiology measured using the carbon-11 radiotracer included: (i) leaf $^{11}CO_2$ fixation; (ii) leaf export of ^{11}C-photoassimilates, (iii) allocation of ^{11}C-photoassimilates belowground; and (iv) root exudation of ^{11}C-photoassimilates.

Positron autoradiography (Typhoon 7000: GE Healthcare, Piscataway, NJ, USA) was used to obtain two-dimensional whole-plant images of the plant shoots and roots. The source leaf section where $^{11}CO_2$ was administered was removed prior to imaging. This data was later used to calculate distributions of radioactivity within targeted tissues using ImageQuant TL 7.0 software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Afterwards, plant tissues were harvested, weighed and counted for radioactivity levels using a Capintec Radioisotope Dose Calibrator CRC-15W (Capintec, Inc, Ramsey, NJ, USA). Radioactivity was corrected for decay using the end-of-bombardment time as time zero. The fresh tissue masses were used to normalize radioactivity amounts enabling us to accurately project ^{11}C-photoassimilate allocation patterns independent of sink mass.

3.2.6 Tissue Extraction

Source leaf tissues exposed to the $^{11}CO_2$ were harvested, weighed and counted in the Capintec Radioisotope Calibrator for radioactivity levels. Tissues were placed into 1.5 mL Eppendorf™ tubes into which 4x w/v of methanol was added. Tissues were fresh ground using a Retsch Miximill MM400 ball and mill grinder (Retsch GmbH, Germany) after which they were briefly vortexed (VWR analog vortex mixer; Sigma-Aldrich Corp. St. Louis, MO, USA) and then sonicated (Branson Bransonic 32; Sigma-Aldrich Corp. St. Louis, MO, USA) in an iced water bath for 10 min with intermittent vortexing to ensure complete mixing. Tubes were centrifuged (Eppendorf Centrifuge 5424) for 2 min at 15,000 rpm and the supernatant removed by pipette. Pellets contained all insoluble components which comprised mostly cell-wall polymers and starch. Filtrates contained small soluble compounds, including soluble sugars and amino acids. Aliquots of the soluble fraction and the total pellet were measured for ^{11}C-radioactivity using a gamma scintillation counter.

3.2.7 ^{11}C- Sugar Analysis

Soluble sugars were separated and analyzed by thin layer chromatography (TLC) (BABST; KARVE; JUDT, 2013). Glass backed NH_2-silica HPTLC-plates (200µm, w/UV254) were used for the sugar separation (Sorbent Technologies, Atlanta, GA, USA). Plates were pre-spotted with sugar standards of glucose, sucrose and fructose for registration of ^{11}C-sugar R_f signatures against those of authentic compounds and then with 1and 2 µL aliquots of radioactive leaf extract using a semi-automatic Linomat 5 sample applicator (Camag Scientific Inc., Wilmington, NC, USA) for high precision of spot size and sample volume. The larger volume of extract was sometimes needed to visualize the ^{11}C-hexose sugars which were usually lower in concentration than sucrose. TLC plates were developed using a mobile phase consisting of 75:25 acetonitrile:water (v/v). Developed plates were imaged using autoradiography (Typhoon FLA 7000) to determine the fraction of each radiolabeled sugar. The Plates were then heat-treated (200^0C for 10 min) to initiate chemical reaction of individual sugars with the amino functionalized silica-support that gave fluorescence under long wavelength (365 nm) UV light. Digital photographs of the fluorescent markers were used to determine the positions of the ^{12}C-sugar standards. ImageQuant TL software 7.0 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used to analyze both the radiographic images to determine the relative amount of ^{11}C within the individual sugars. ^{11}C-Sugars were placed on a relative distribution of ^{11}C-soluble activity and later corrected to reflect total ^{11}C-activity within the targeted tissue using ^{11}C-soluble and ^{11}C-insoluble fractions.

3.2.8 ^{11}C-Amino Acid Analysis

A 50 µL volume of the soluble extract was delivered into a 0.5 mL Eppendorf™ tube (Fisher Scientific, Inc., Pittsburgh, PA, USA) and mixed with an equal volume of o-phthalaldehyde amino acid derivatizing reagent (OPA: Sigma-Aldrich Inc., St. Louis, MO, USA) containing 0.1% (v/v) mercaptoethanol and 0.1% (v/v) sodium hypochlorite. The mixture was vortexed and then allowed to react at ambient temperature for 3 min. Primary amino acids are readily converted into iso-indole derivatives by OPA enabling their separation with reversed-phase high-performance liquid chromatography and quantification

by fluorescence detection (CHOW; ORENBERG; NUGENT, 1987). A 20 μL volume of the derivatized mixture was injected onto a reversed-phased analytical HPLC column (Phenomenex, Torrance, CA, USA: Ultramex™ C18, 10 μm particle size, 250 × 4.6 mm i.d.) using a pre-column gradient mixer (Isco, Lincoln, NE, USA) and a mobile phase comprised of A (DI water), B (0.01 M potassium phosphate (monobasic) solution buffered at pH 6.5), and C (methanol). At injection, the mobile phase (1.8 mL min^{-1}) was sustained at 75% A: 25% B for 5 min and then programmed to attain 20% B: 80% C by 30 min using a Knauer solvent gradient mixer (Sonntek Inc., Upper Saddle River, NJ, USA). Elution profiles were calibrated against standards using a fluorescence detector (Hitachi FL Detector L-2485). The outlet of the fluorescent detector was connected in series to a NaI gamma radiation detector (Ortec Inc, Oak Ridge TN, USA) that enabled direct measurement of the amount of radioactivity associated with each substrate eluting the column. Analog outputs from detectors were fed to a chromatography data acquisition station (SRI Instruments, Torrance, CA, USA). The radiation detector was also cross-calibrated against the gamma detector that was used to measure the soluble and insoluble extract fractions so that ^{11}C-amino acids could be correlated to total ^{11}C-activity levels within the plant (HANIK et al., 2010).

3.2.9 Statistical Analysis

Data was subjected to the Student t-test assuming for unpaired samples assuming an unequal variance and statistical significance assigned for $P \leq 0.050$.

3.3 RESULTS

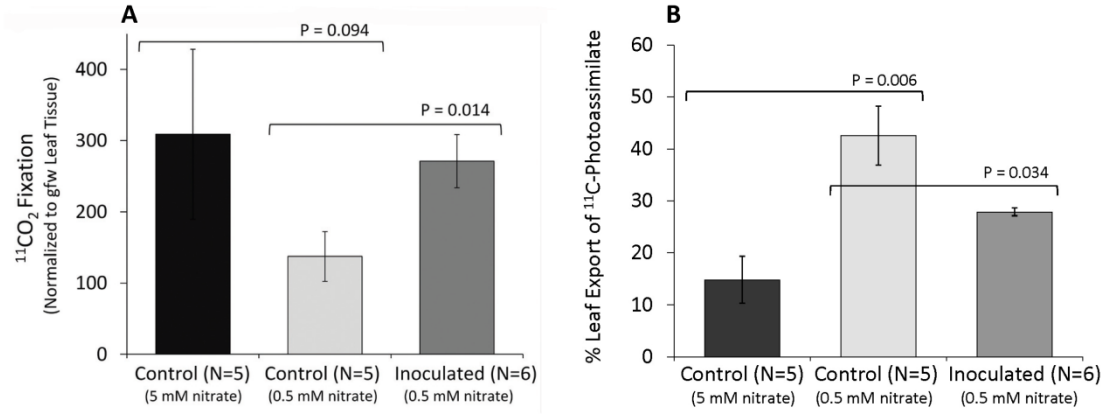
3.3.1 Bacterial counting

Estimated bacterial numbers per unit root were increased by inoculation in both total and internal colonization per gram of fresh weight root (gfw^{-1}). After 15 days of inoculation, plants presented total grown about 10^6 to 10^8 $cfu.gfw^{-1}$ for *A. brasilense* and *H. seropedicae*, respectively. Additionally, internal *H. seropedicae* colonization showed 10^6 $cfu.gfw^{-1}$.

3.3.2 Changes in Whole-Plant Physiology

Control plants grown under nitrate limitation showed a trend of reduced $^{11}CO_2$ fixation (Fig. 3.1A) relative to control plants grown under normal nitrate conditions, though the level of significance was weak (*P*= 0.094). However, inoculated plants grown under nitrate limitation showed a significant increase in fixation levels (*P*=0.014) over uninoculated control plants grown under similar conditions bringing fixation back to those levels observed for controls grown under normal nitrate.

Figure 3.1: The physiological responses on the distribution of $^{11}CO_2$ fixation and ^{11}C-photosynthate of plants of *Setaria viridis*, grown under normal nitrate levels (Control 5 mM N), low nitrate levels (Control 0.5 mM N), and inoculated samples under low nitrate levels (Inoculated 0.5 mM N). A: Percentage of $^{11}CO_2$ fixed by the LL (load leaf) normalized to gram fresh weight (gfw) enclosed within the leaf cell. B: Percentage of ^{11}C-photosynthate exported by the LL following treatment. The bars are mean +/- SE. The significant difference is shown by *P* value.

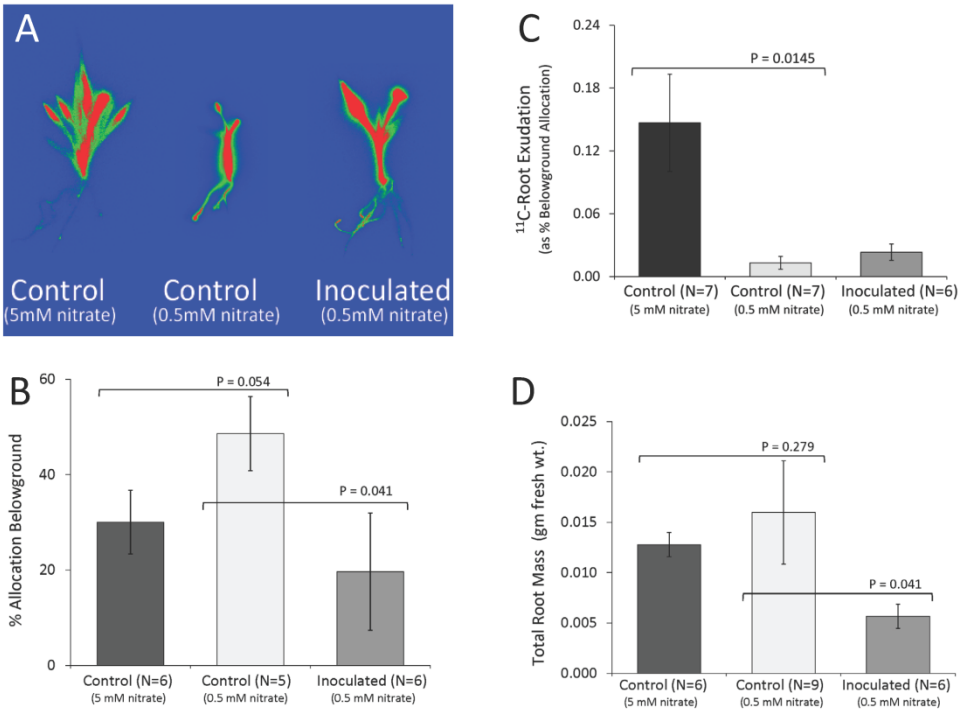


Though $^{11}CO_2$ fixation was reduced for uninoculated plants grown under nitrate limitation, leaf export of ^{11}C-photoassimilates (Fig. 3.1B) was significantly higher in these plants (*P*=0.006) relative to control plants grown under normal nitrate conditions. Inoculated plants, however, showed a significant reduction in ^{11}C-photoassimilate export (*P*=0.034) relative to uninoculated controls grown under the same nitrate level indicating a shift in resource transport dynamics back to a normal unstressed state.

Radiographic images (Fig. 3.2A) reflecting the bio-distribution of those ^{11}C-photoassimilates exported from the load leaf showed a trend of increased radiotracer distribution belowground for control plants grown under nitrate limitation. This trend was

quantitatively verified using "cut-and-count" techniques to measure the amount of radioactivity in the different tissues (Fig. 3.2B), and showed that plants grown under nitrate limitation exhibited a significant increase in belowground allocation (*P*=0.054) relative to normal control plants. This trend was again reversed (*P*=0.041) when plants were inoculated with bacteria, demonstrating once again that the presence of the bacteria re-instated "normal" plant physiological responses.

Figure 3.2: Changes in $^{11}CO_2$ export and distribution by LL (load leaf) in Whole-Plant of *S. viridis* grown under normal nitrate levels (Control 5 mmol N), low nitrate levels (Control 0.5 mmol N), and inoculated samples under low nitrate levels (Inoculated 0.5 mmol N) followed to belowground responses as allocation, exudation and root mass. A: Positron autoradiograph image of the shoot and root tissues without the LL. Blue coloration indicates low radioactivity with increasing radioactivity for yellow and then red. B: Percentage of ^{11}C-photosynthate allocated to *S. viridis* roots. C: Percentage of ^{11}C-root exudation. D: Total root mass. All bars are mean ± SE The significant difference is shown by *P* value.



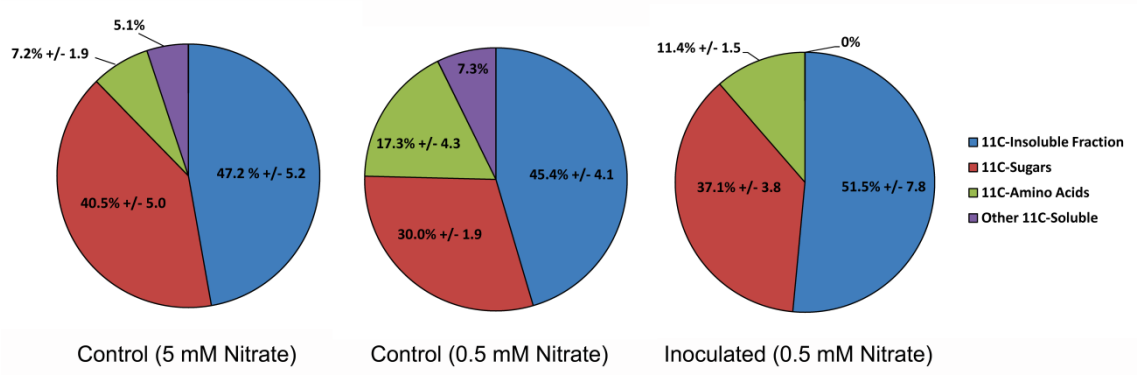
Furthermore, we observed a significant reduction in ^{11}C-photoassimilate exudation (Fig. 3.2C) from control roots under nitrogen limitation, as well as from bacteria association relative to normal nitrogen roots (*P*=0.014). Although not significant *(P*=0.279), there was a systematic increase observed in total root mass (gfw) of uninoculated control plants grown under nitrate limitation (Fig. 3.2D) as compared with control plants grown under normal nitrate conditions. The slight increase in control plant root mass under nitrate limitation was observed to be due to increased primary root length and not due to increased branch root patterning. However, this trend of increased root mass was reversed significantly in

inoculated plants (P=0.041). Though total root mass was lower under these conditions a slight increase in branch root patterning was observed.

3.3.3 ^{11}C Metabolism

Metabolic fluxes of new carbon, reflected as ^{11}C, were traced through and into the various pools of soluble and insoluble (ie. storage) substrates (Fig. 3.3) of load leaf tissue 1 hr after exposure to $^{11}CO_2$. Results showed no significant change in the insoluble ^{11}C-fractions measured across the three growth conditions (control, 5 mM nitrate; control, 0.5 mM nitrate; bacteria inoculated, 0.5 mM). However, nitrate limitation of uninoculated plants resulted in a decrease (from 40.5 ± 5.0% total ^{11}C-activity to 30.0 ± 1.9%) in the soluble ^{11}C-sugar pool relative to normal controls that was significant (P=0.052). This response was compensated for by an increase in the ^{11}C-amino acid pool (from 7.2 ± 1.9% to 17.3 ± 4.3%) that was marginally significant (P=0.066).

Figure 3.3: The pie chart shows pools of ^{11}C-sugars (red), ^{11}C-amino acids (green), ^{11}C-insoluble (blue), and ^{11}C-other soluble (purple) on LL (load leaf) of *S.viridis* under normal nitrate levels (Control 5 mmol N), low nitrate levels (Control 0.5 mmol N), and inoculated samples under low nitrate levels (Inoculated 0.5 mmol N). The significance is shown as percentage of pools with P<0.05.

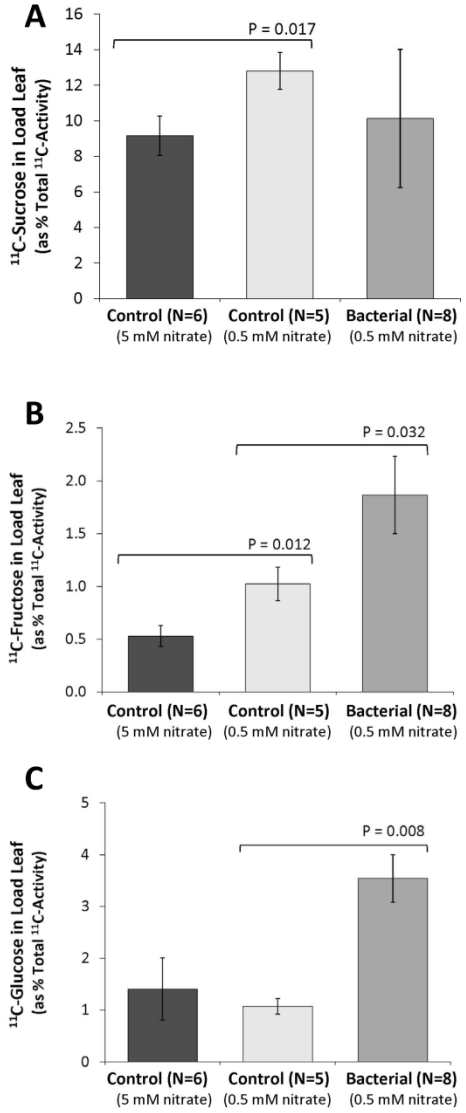


Results further demonstrated that bacteria inoculated plants grown under nitrate limitation exhibited similar metabolic behavior as control plants grown under normal nitrate conditions. Specifically, an increase in the ^{11}C-soluble sugar pool (from 30.0 ± 1.9% to 37.1 ± 3.8%) was observed relative to nitrate limited control plants, though this change was only marginally significant (P=0.082). This trend was also compensated for by a reduction in the

^{11}C-amino acid pool (from 17.3 ± 4.3% to 11.4 ± 1.5%), though the change was not considered significant (P=0.148). Taken together, these observed changes in new carbon partitioning into total soluble ^{11}C-sugar and ^{11}C-amino acid pools was seen as a re-instatement of "normal" plant metabolic behavior under nitrate limitation that was invoked by the presence of bacteria.

A closer inspection of individual ^{11}C-sugars, including ^{11}C-sucrose, ^{11}C-glucose and ^{11}C-fructose, revealed subtle effects of the growth conditions (Fig 3.4A-C) on labeled sugar levels. Control plants grown under nitrate limitation exhibited significant increases in ^{11}C-sucrose (P=0.017) and ^{11}C-fructose (P=0.012) levels, but no change in the ^{11}C-glucose level relative to normal controls. Inoculated plants exhibited significant increases in both ^{11}C-fructose (P=0.032) and ^{11}C-glucose (P=0.008) levels balanced against what we perceived as a decreasing level of ^{11}C-sucrose, though the error in the ^{11}C-sucrose data was too large to draw any firm conclusions about growth effects.

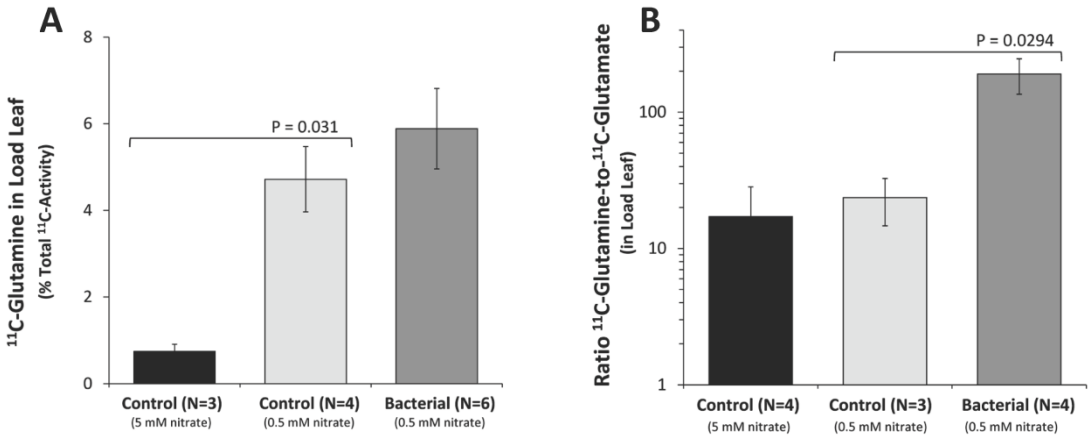
Figure 3.4: Metabolic partitioning in leaves of *Setaria viridis* inoculated with a mix of nitrogen fixing bacteria *A. brasilense* and *H. seropedicae*, under normal nitrate levels (Control 5 mmol N), low nitrate levels (Control 0.5 mmol N), and inoculated samples under low nitrate levels (Inoculated 0.5 mmol N) into [^{11}C] sugars for LL after 1h pulse of $^{11}CO_2$ administration. A: Total activity of ^{11}C-Sucrose. B: Total activity of ^{11}C-Fructose and C: Total activity of ^{11}C-Glucose All bars are means +/- SE. The significant difference is shown by *P* value.



Furthermore, a closer inspection of the individual ^{11}C-amino acids contributing to the measureable pool of ^{11}C-amino acids (data not shown) revealed that ^{11}C-glutamine exhibited significant changes as a function of the growth conditions (Fig 3.5A). Specifically, control plants grown under nitrate limitation showed significantly higher levels of ^{11}C-glutamine than normal controls (*P*=0.031). This elevated level of ^{11}C-glutamine remained unchanged in the presence of the bacteria. A plot of the ratio of ^{11}C-glutamine-to-^{11}C-glutamate (Fig. 3.5B) revealed more subtle features of the effects of growth conditions on host nitrogen metabolism. For control plants grown under nitrate limitation this ratio was unchanged from normal

control plants. However, for inoculated plants grown under nitrate limitation, a significant increase in the ^{11}C-glutamine-to-^{11}C-glutamate ratio was observed (P=0.029).

Figure 3.5: Yields of ^{11}C-glutamine amino acid within the LL (load leaf) of *S. viridis* associated with *A. brasilense* and *H. seropedicae*, under normal nitrate levels (Control 5 mmol N), low nitrate levels (Control 0.5 mmol N), and inoculated samples under low nitrate levels (Inoculated 0.5 mmol N). A: Total activity of ^{11}C-glutamine amino acid in LL. B: Ratio of ^{11}C-glutamine-to-^{11}C-glutamate amino acids within the load leaf. All bars are means ±SE. The significant difference is shown by *P* value.



3.4 DISCUSSION

In general, past research addressing associative effects of PGPR have primarily focused on certain phenotypic traits of their respective hosts including plant biomass and crop yield or seed count (BODDEY, ROBERT M., 1995; GYANESHWAR et al., 2002; HUNGRIA, MARIANGELA et al., 2010). Here we present what is believed to be the first detailed mechanistic study that addresses the physiological and metabolic basis for understanding PGPR associations in the context of host ability to sustain healthy growth under nitrogen limitation.

Our studies in *S. viridis* grass as a model system addressed two very basic attributes of plant physiology that help shape plant growth characteristics. These attributes included: (i) carbon input via CO_2 fixation; and (ii) allocation of carbon-based resources belowground. The first was highly quantifiable in our studies using radioactive $^{11}CO_2$. The second attribute was further refined by being subdivided into three highly quantifiable metrics that included leaf export of mobile ^{11}C-based resources, their transport belowground to roots, and their transference out of the roots to the surrounding rhizosphere. Taken together, information from

these four attributes was leveraged to provide a comprehensive view of how plants use their carbon resources when subjected to three different environmental growth regimes encompassing: (i) *S. viridis* grown under normal nitrate conditions (5 mM NO_3^-); (ii) *S. viridis* grown under nitrate-limitation (0.5 mM NO_3^-); and (iii) bacterial inoculated *S. viridis* grown under nitrate-limitation (0.5 mM NO_3^-).

Our results from testing uninoculated plants subjected to nitrate limitation clearly suggest that the plants become stressed and respond accordingly. For example, their lowered CO_2 fixation rate is a characteristic response to the limited nitrogen supply and reflects the plant's attempt to re-acclimate by balancing its carbon input to the supply of nitrogen available (for a review see; ZHENG, 2009). Furthermore, their increased export of available mobile ^{11}C-photoassimilates from source leaves to roots was an attempt to channel what limited carbon resources were available belowground in support of root growth. This observation correlates well with previous findings that plants subjected to nutrient and/or water stress invested more carbon resources to their root system (BLOOM; CHAPIN; MOONEY, 1985; KOBE; IYER; WALTERS, 2010; POORTER et al., 2012) as a compensatory mechanism to increase root growth for enhanced foraging capability. Indeed, we observed a slight enhancement in the root length under these growth conditions and a significant reduction in root exudation implying that transported carbon belowground was being used for compensatory growth.

Additionally, the sustained reduced levels observed in ^{11}C-root exudation for inoculated plants relative to normal controls may be due strictly to biotic factors. That is, since root growth is no longer elevated, limitations in ^{11}C-transferance from roots-to-rhizosphere may be tightly regulated as a way for the host to provide nutrient resources to the bacterial populations that have colonized its root system. Comparable results have been described for bacterial inoculation of maize after ^{14}C application to shoots (SCHULZE; POSCHEL, 2004). In this prior work, ^{14}C-root exudates were significantly reduced for plants inoculated with either *Pantoea* or *Pseudomonas* bacteria.

Finally, we took an in-depth look at how plants responded metabolically to the three imposed growth regimes. As noted earlier, abiotic stress elicited by nitrate limitation resulted in a significant reduction of new carbon partitioning (as ^{11}C) into soluble sugars that formed a portion of the load leaf's photoassimilate pool, though we note there was a significant increase in the soluble ^{11}C-sucrose fraction of that pool. Sucrose is highly transportable between tissues/organs of the plant which may account for why we saw an increase in leaf ^{11}C-photoassimilate export from the load leaf. Furthermore, this action of down-regulating

new carbon partitioning into the general sugar fraction was compensated for by a commensurate increase in new carbon partitioning into amino acids. Reprogramming of primary metabolism has been documented by us in the past where defense elicitation, triggered by a combination of mechanical wounding and exogenous jasmonate application to tobacco leaves, resulted in significant shifts of ^{11}C-partitioning into amino acids (HANIK et al., 2010) at the expense of ^{11}C-soluble sugars within hours of treatment. This rapid reprogramming of plant metabolism was rationalized on the grounds that plants often manufacture specialized secondary defense compounds in response to herbivory requiring rapid mobilization of nitrogen resources. In the present study, however, we can rationalize this same metabolic behavior on the grounds that changes in root growth and/or root system architecture are triggered by increased hormonal activity (AGTUCA et al., 2013) requiring increased mobilization of nitrogen resources to sustain not only their biosynthesis (BRAZELTON et al., 2008; PHILLIPS et al., 2004), but also that of cell-wall lignin during growth.

Metabolic bioassays carried out on nitrate limited inoculated plants as a whole showed a systematic trend toward re-instatement of "normal" partitioning patterns for new carbon fluxes into soluble sugar and amino acid fractions of the load leaf's photoassimilate pool. However, within these soluble fractions inoculated plants did exhibit some distinct signatures. For example, significantly higher levels of radiolabeled monosaccharide sugars were observed in inoculated source leaves (as ^{11}C-fructose and ^{11}C-glucose) relative to uninoculated control plants. This is an important feature to consider since *H. seropedicae,* an endophytic bacterium, is capable of sustainable growth on monosaccharidic sugars including D-glucose and D-fructose (PEDROSA et al., 2011). We suspect that this action of fine tuning sugar metabolism by the host may reflect an effort to provide essential nutrient resources to the bacterial colonies residing within it. This theory is further supported by our observation that root exudation is significantly reduced in the presence of bacteria.

Furthermore, an in-depth analysis at specific radiolabeled amino acids revealed that ^{11}C-glutamine levels were also significantly elevated in inoculated plants, as they were for uninoculated nitrate limited plants. We surmise that the elevation in ^{11}C-glutamine levels for uninoculated nitrate limited plants was a reflection of the general increased flux of new carbon into the greater amino acid pool, and indeed the ratio of ^{11}C-glutamine-to-^{11}C-glutamate under these conditions was unchanged from that of "normal" control plants, suggesting that glutamine metabolism was unaffected by the nitrate limitation. However, the persistent elevation in ^{11}C-glutamine levels in inoculated plants, even after the total ^{11}C-amino

acid pool was reduced, suggests that host glutamine metabolism was inhibited in the presence of the bacteria. Indeed, the significant increase in ^{11}C-glutamine-to-^{11}C-glutamate under these conditions supports this theory.

This observation may have strong biological relevance to the persistent theory for why *S. viridis* is especially responsive to PGPR, while other C_4 grasses are not. Here we suggest that *S. viridis*, and especially the A10.1 genotype, is better equipped to harness biological nitrogen from the actions of the N_2-fixing bacteria owing to the fact that its ability to take up nitrate from the soil has been severely down-regulated. This theory is based, in part, on one very important fact that both glutamine and glutamate play important roles as signaling molecules in the regulation root nitrate permeases and foliar nitrate reductase (FORDE; LEA, 2007). That is, elevated levels of both can have adverse effects on nitrate uptake. Studies are presently underway that will shed more light on this subject. Leveraging radioactive ^{13}NN ($t_{½}$ 9.97 min) tracer we will be uniquely positioned to carry out highly sensitive and highly quantitative measurements showing direct evidence for host nitrogen input *via* the actions of N_2-fixing bacteria. Additional studies using $^{13}NO_3^-$ are also essential to provide the supportive data that is needed showing the down-regulation of host nitrate uptake. Finally, metabolic flux assays utilizing high specific activity ^{11}C-L-glutamine are already underway to fine-tune our understanding of root glutamine and glutamate metabolism within targeted zones that overlap with areas of high bacterial colonization.

In conclusion, the present work leveraging carbon-11 radiotracer technology shows very strong evidence that bacterial colonization of host *S. viridis* roots can alter whole-plant physiological and metabolic processes in a way that better supports the growth of the bacterial colonies as well as that of their host in a purely synergist association.

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CAPÍTULO 4

USO DO RADIOISÓTOPO [^{13}N]N_2 ADMINISTRADO EM RAÍZES DE *Setaria viridis* PARA AVALIAR A ASSIMILAÇÃO DE NITROGÊNIO FORNECIDO POR BACTÉRIAS FIXADORAS DE NITROGÊNIO

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Resumo

Rizobactérias diazotróficas são uma ferramenta biotecnológica promissora para melhorar a produtividade agrícola por meio da fixação biológica de nitrogênio e promoção de crescimento vegetal. Uma possível aplicação desta tecnologia e na melhoria agronômica de culturas bioenergéticas, que são direcionados para a produção em terras marginais. Muitos trabalhos atestam os efeitos de promoção de crescimento de plantas por várias bactérias endofíticas (HUREK, T.; REINHOLD-HUREK; VAN MONTAGU; et al., 1994; JAMES, E. K.; OLIVARES, F. B. L., 1998; YANNI et al., 1997), incluindo aumentos significativos na produtividade da cultura (CHARYULU et al., 1985; DOBBELAERE, SOFIE et al., 2001; OKON, YAACOV; LABANDERA-GONZALEZ, 1994; PEDRAZA, RAÚL O. et al., 2009). Uma vez que estas bactérias fixam nitrogênio atmosférico, assume-se que a fixação biológica de nitrogênio é responsável por aumentar o crescimento da planta. No entanto, poucos estudos têm fornecido dados convincentes para fixação em planta e menos ainda para a incorporação de nitrogênio fixado pela planta hospedeira. A maior parte, é exemplificado por estudos de observação em espécies de plantas que são, em grande parte, recalcitrante para estudos do mecanismo e genéticos. *S. viridis* é uma gramínea C_4, intimamente relacionada com *swtichgrass*, e tem sido proposta como uma planta modelo experimentalmente tratável para estudos relacionados a culturas de bioenergia. Para este estudo, plantas de *Setaria viridis* foram inoculadas com duas bactérias fixadoras de nitrogênio, já caracterizadas, *Herbaspirillum seropedicae e Azospirillum brasilense*. O estudo mais significativo, utilizando o radioisótopo $^{13}N_2$, mostrou níveis significativos de nitrogênio fixado e, ainda, o movimento e a incorporação desse nitrogênio pela planta. Os dados indicam claramente que *S. viridis* é um sistema modelo de planta geneticamente tratável, que mostra a fixação e a incorporação de N_2 após a inoculação com as bactérias. Isso abre as portas para futuros estudos para elucidar os mecanismos de fixação de nitrogênio por bactérias associativas e, finalmente, a promessa de utilização de tais sistemas para a melhoria agronômica.

4 USE OF [^{13}N]N_2 RADIOTRACER ADMINISTERED TO ROOTS OF *Setaria viridis* TO EVALUATED THE NITROGEN ASSIMILATION PROVIDED BY NITROGEN FIXING BACTERIA

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Abstract

Diazotrophic rhizobacteria are a promising biotechnological tool to improve agricultural productivity through biological nitrogen fixation and plant growth promotion. One possible application of this technology would be in the agronomic improvement of bioenergy crops, which are targeted for production on marginal lands. A large body of literature attests to the plant growth promotion effects of various endophytic bacteria (HUREK, T.; REINHOLD-HUREK; VAN MONTAGU; et al., 1994; JAMES, E. K.; OLIVARES, F. B. L., 1998; YANNI et al., 1997), including significant increases in crop yield (CHARYULU et al., 1985; DOBBELAERE, SOFIE et al., 2001; OKON, YAACOV; LABANDERA-GONZALEZ, 1994; PEDRAZA, RAÚL O. et al., 2009). Since these bacteria fix atmospheric nitrogen, an obvious assumption is that biological nitrogen fixation is responsible for enhancing plant growth. However, few studies have provided convincing data for fixation *in planta* and even fewer for incorporation of fixed nitrogen by the plant host. The field is exemplified by observational studies in plant species that are largely recalcitrant to detailed genetic and mechanistic studies. *S. viridis* is a C_4 grass, closely related to swtichgrass, and has been suggested as an experimentally tractable model plant for studies related to bioenergy crop plants. For this study we inoculated *Setaria viridis* with two well characterized, nitrogen fixing bacteria, *Herbaspirillum seropedicae* and *Azospirillum brasilense*. The most significant, $^{13}N_2$ tracer studies showed significantly levels of nitrogen fixed and, more

importantly, movement and incorporation of this nitrogen by the plant. The data clearly indicate that *S. viridis* is a genetically tractable plant model system, which shows N_2 fixation and incorporation upon bacterial inoculation. This opens the door to future studies to define the mechanisms of associative nitrogen fixation and ultimately the promise of utilization of such systems for agronomic improvement.

Keywords $^{13}N_2$ isotope. *Setaria viridis***.** *Azospirillum brasilense*. *Herbaspirillum seropedicae*. Plant-bacteria interaction. Nitrogen fixation.

4.1 INTRODUCTION

An important component of any non-leguminous cropping system, especially for bioenergy crops, is nitrogen management. Fossil fuels are used to produce nitrogen fertilizer; therefore, a reduction in nitrogen inputs can significantly impact the energy balance of the resulting biofuel. Tjepkema and Burris (TJEPKEMA; BURRIS, 1976) reported that switchgrass can gain some nitrogen through biological nitrogen fixation (BNF) mediated by plant growth-promoting rhizobacteria (PGPR). Similarly, *Miscanthus* does not appear to require N fertilizer under some conditions. For example, one fourteen-year study found that varying levels of N fertilization had no effect on *Miscanthus* yield (CHRISTIAN; RICHE; YATES, 2008). PGPR that fix nitrogen have been isolated from *M. gigantaeus* roots (ECKERT et al., 2001). Indeed, Carroll and Somerville (CARROLL; SOMERVILLE, 2009) postulated that the variation in reports of *Miscanthus* response to N fertilization was likely due to variation in the association with such nitrogen-fixing PGPR.

PGPR colonize roots and engage in associative symbiosis with various host plants, including bioenergy grass species (SANTI et al., 2013). In most cases, the mechanism of plant growth promotion is unknown. In selected cases, plant growth promotion is attributed to antagonism toward phytopathogens (RAAIJMAKERS et al., 2009) and/or the induction of plant resistance (VERHAGEN et al., 2004). Other PGPR may act mostly by phytostimulation (e.g., release of phytohormones (RICHARDSON et al., 2009)). Several nitrogen-fixing PGPR have been identified as endophytes of grass species, including *Azoarcus* spp. in Kallar grass and rice (HUREK, T.; REINHOLD-HUREK; VAN MONTAGU; et al., 1994; REINHOLD et al., 1986), *Herbaspirillum seropedicae* in sugarcane (JAMES, E. K.; OLIVARES, F. B. L., 1998) and sorghum (JAMES et al., 1997), and *Gluconacetobacter diazotrophicus* in sugar cane (JAMES et al., 1994). Unlike rhizobia that form an intimate intracellular symbiosis with their legume hosts, PGPR do not induce the formation of observable plant structures. They are also usually not major components of the soil microflora (JAMES, E. K.; OLIVARES, F. B. L., 1998; REINHOLD-HUREK, BARBARA; HUREK, THOMAS, 1998). These nitrogen-fixing endophytes infect at the emergence of lateral roots and in the zone of elongation and differentiation above the root tip. After infection, the bacteria colonize the outer root cell layers and the root cortex. They can also gain access to the vascular tissue. These associations are strictly defined by the lack of any evidence of intracellular infection (JAMES, E. K.; OLIVARES, F. B. L., 1998; REINHOLD-HUREK, BARBARA; HUREK, THOMAS, 1998).

However, very high numbers of PGPR in roots have been reported (i.e., $\leq 10^8$/ gram root dry weight) and with no observable disease symptoms (BARRAQUIO; REVILLA; LADHA, 1997; REINHOLD et al., 1986).

Many PGPR are capable of BNF. However, the role of BNF in plant growth promotion has not been well documented. The most publications simply the report the presence of nitrogen fixing PGPR or perhaps the *in planta* expression of bacterial nitrogenase protein or genes in endophytes, for example *Azoarcus* sp. in rice (EGENER; HUREK; REINHOLD-HUREK, 1999; HUREK, T.; REINHOLD-HUREK; TURNER; et al., 1994). Only a few rare studies have provided convincing data for fixation *in planta* and even fewer for incorporation of fixed nitrogen by the plant host. Notable positive examples include the interaction between *G. diazotrophicus* and sugar cane (SEVILLA et al., 2001), *Azoarcus* sp. strain BH72 and Kallar grass (HUREK, THOMAS et al., 2002), or *Klebsiella* sp. and wheat (INIGUEZ; DONG; TRIPLETT, 2004). However, even in these cases, the estimates are that the amount of nitrogen fixed would provide little or no contribution to the overall nitrogen demand. In contrast, some studies conducted in the field with wild, grass species suggest that 30% or more of the plant nitrogen demands can be provided by BNF (BODDEY, R. M.; VICTORIA, 1986; MORAIS et al., 2012), attesting to the promise of this approach. What is clearly needed is a tractable experimental system that exhibits appreciable levels of associative nitrogen fixation in which more detailed, mechanistic studies can be conducted.

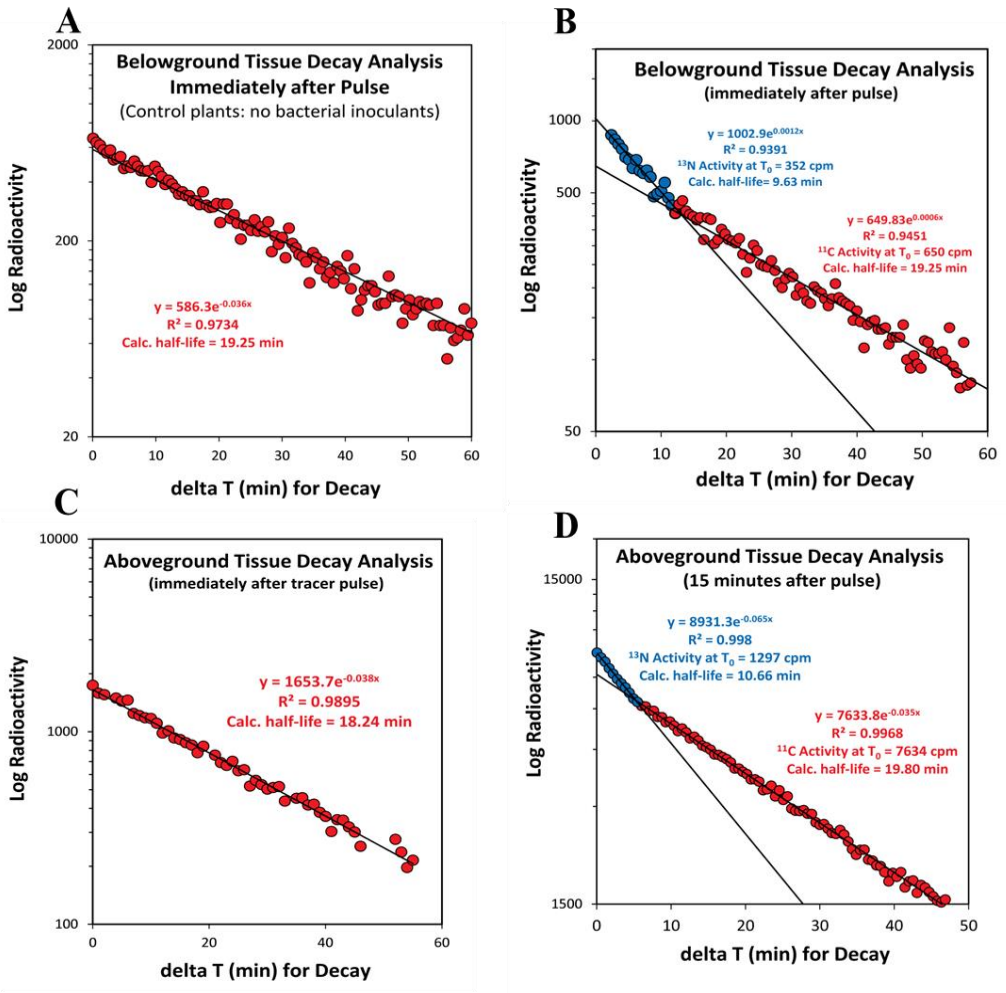
4.2 RESULTS AND DISCUSSION

We inoculated *S. viridis* A10.1, the genotype currently being used by the Department of Energy-Joint Genome Institute for genome sequencing (http://www.jgi.doe.gov), with two beneficial diazotrophic bacteria, *Azospirillum brasilense* and *Herbaspirillum seropedicae*; both of which have been utilized as commercial inoculants, mainly in Latin America. *H. seropedicae* is a diazotrophic and endophytic bacterium, which belongs to the *Beta-proteobacteria* group. In contrast, *A. brasilense* is a member of the *Alpha*-proteobacteria and its growth is confined to the plant root surface (BASHAN; HOLGUIN; DE-BASHAN, 2004; DOBBELAERE, SOFIE et al., 2001; TARRAND JJ; KRIEG NR; J., 1978).

Demonstration of nitrogenase gene expression is not sufficient to conclude that BNF is occurring or, more importantly, that fixed nitrogen is being used by the plant. Hence, to address these issues, we measured the fixation and incorporation of $^{13}N_2$ gas by plants either

uninoculated or inoculated with *A. brasilense* and *H. seropedicae.* Radiographic images of inoculated whole plants revealed that the root systems acquired a measurable amount of radioactivity though it was not possible to ascertain the nature of the isotopic signature from the images (data no shown). We also noted a significant amount of radioactivity fixed in photosynthetically active tissues that were unavoidably captured beneath the stem flange. Follow-up decay analysis (Figure 4.1A-D) on those targeted tissues verified that the radioactivity isolated in the aerial portions of the plant immediately after the tracer pulse only had a carbon-11 signature (Figure 4.1A) attributable to fixation of the small amount of $^{11}CO_2$ in the pulse. Roots from the same inoculated plants, however, revealed a mixture of nitrogen-13 and carbon-11 isotopes when subjected to this analysis immediately following the tracer pulse (Figure 4.1B).

Figure 4.1: Decay analysis of ^{13}NN activity administered in roots of *Setaria viridis* uninoculated and inoculated with *A.brasilense* and *H.seropedicae*. A: Radioactivity in belowground tissue of control plants right after $^{13}N_2$ pulse shown only carbon-11signature (red dots). B: Radioactivity in belowground tissue of inoculated plants right after $^{13}N_2$ pulse shown carbon-11 (red dots) and nitrogen-13 (blue dots) signature. C: Radioactivity in aboveground tissue of control plants right after $^{13}N_2$ pulse shown only the carbon-11 signature (red dots). D: Radioactivity in aboveground tissue of inoculated plants 15 minutes after $^{13}N_2$ pulse shown carbon-11 (red dots) and nitrogen-13 (blue dots) signature.



Isotopic identification was based on a strong correlation between the experimentally derived half-lives and the published values. To verify that this nitrogen-13 signature was the result of bacterial N_2 fixation, roots from non-inoculated control plants were subjected to this analysis. Results showed only a carbon-11 signature (Figure 4.1C). Finally, to distinguish our work from prior studies that measured nitrogenase enzyme activity using the acetylene reduction assay, we applied decay analysis to the aerial portions of the plant 15 min after the pulse, during which time the plant was subjected to high illumination (1500 µmol m^{-2} sec^{-1}) as a means to promote the plant's water transpiration stream. By monitoring the radioactivity profile in the lower stem area, it was obvious that a measurable amount of radioactivity was

transported from roots-to-shoots over this 15 minute time course. Furthermore, decay analysis performed on aerial tissues after 15 minutes (Figure 4.1D) revealed a strong nitrogen-13 signature along with carbon-11 suggesting that some portion of the fixed $^{13}N_2$ ends up in biological transport within the host plant.

Two additional studies were conducted in order to better understand the source of the root carbon-11 radioactivity. In one study, we subjected an inoculated plant to darkness, and then detached the aboveground tissues (sealing the base of the stem with lanolin) just prior to administering a pulse of tracer to the soil column and remaining roots. Decay analysis of the root system immediately after the pulse revealed a strong nitrogen-13 and carbon-11 signature (Table 4.1). In the second study, we compared the effect of the light and dark cycles on the isotopic composition fixed within the root system of both inoculated and non-inoculated intact plants. Results in Table 1 were tabulated as disintegrations per minute (dpm) of radioactivity for each isotope component that was isolated from the decay analysis. Individual experimental results were tabulated per treatment type along with the means and standard deviations. Aside from the clear evidence that $^{13}N_2$ fixation occurs only when plants are inoculated with bacteria, our results also show that $^{13}N_2$ is not subject to diurnal variation.

Table 4.1: Decay analysis of ^{13}N-activity and ^{11}C-activity conducted on root system of *S. viridis* under low nitrate level (0.5mmol N) using control and inoculated plants. The experiments were performed in the light presence and also in the dark. The decay was measured by disintegrations per minute (dpm).

Condition	Cycle	Root ^{13}N Activity (dpm)[a]	Root ^{11}C Activity (dpm)
Inoculated	Light	929	2874
Inoculated	Light	1342	5652
Inoculated	Light	1313	5355
	Mean ± SD	**1195 ± 231**	**4677 ± 1525**
Inoculated	Dark	1203	2206
Inoculated	Dark	1000	3542
Inoculated	Dark	1087	2973
	Mean ± SD	**1097 ± 102**	**2907 ± 670[b]**
Control (non-inoculated)	Light	0	3681
Control (non-inoculated)	Dark	0	3468
Inoculated	Detached Root	671	1942

We calculated fractional $^{13}N_2$ fixation values of 15 ± 3 ppb (light) and 13 ± 1 ppb (dark), which equates to a daily N_2 fixation rate of 125 ± 36 nmole/day. On the other hand,

$^{11}CO_2$ fixation by roots was seen to be significantly higher during the light cycle (P=0.0073). These values provide the first hard evidence demonstrating incorporation of fixed nitrogen into the host plant through biological transport of nitrogen-13 to aerial portions of the plant. Radioactivity levels in the shoots were low preventing a more in-depth analysis for chemical identification. Even so, our fractional fixation reflects a daily rate of N_2 uptake by the bacteria of 125 ± 36 nmoles N_2/day. We estimate from biological transport that approximately 30% of this value is assimilated by the host. Small grasses, most particularly small maize, require between 150-350 nmoles of NO_3^{-1}/day for routine growth. Hence, our calculations indicate that BNF can provide a significant amount of assimilated nitrogen for *S. viridis* plant growth. Potentially higher levels of nitrogen could be provided with further optimization of the system.

Interestingly, our results also showed that $[^{13}N]N_2$ fixation was not impacted by diurnal effects. This is consistent with what was observed in root nodules of soybean plants (WEISZ; SINCLAIR, 1988). There, nitrogenase activity exhibited strong cyclic behavior mimicing the daily fluctuations in soil temperature, but when that variable was removed, it remained fairly constant throughout the day. Our studies were conducted at constant temperature, as well.

4.3 METHODS

4.3.1 Bacteria growth condition

H. seropedicae strain RAM4 expressing the DsRed gene fusion (MONTEIRO et al., 2008) was grown in liquid NFb-Malate medium (KLASSEN et al., 1997) and *A. brasilense* strain FP2 *nifH:gusA* was grown in liquid NFb-Lactate medium (MACHADO et al., 1991), 50 mmol/L of phosphate and 20 mmol/L of NH_4Cl_2 was added to both media, which arehereafter called NFbHPN-malate or NFbHPN-lactate. The bacterial cultures were grown at 30°C with shaking at 130 rotations per min (rpm). Streptomycin at a final concentration of 80 μg/mL was added to both cultures. Kanamycin at a final concentration of 200 μg/mL was added to *H. seropedicae* cultures. Tetracycline and kanamycin were added to *A. brasilense* cultures at a final concentration of 10 μg/mL and 50 μg/mL, respectively.

4.3.2 *S. viridis* seed sterilization and germination

Seeds of *S. viridis* A10.1 genotype were first sterilized with 6% (v/v) bleach plus 0.1% (v/v) Tween 20 for 3 min and rinsed five times with sterile distillated water. The sterile seeds were plated with the embryos facing upwards in Petri dishes filled with Hoagland's fortified agar gel. Agar gels were prepared out of 3 L of de-ionized water, 4.9 g Hoagland modified basal salt mixture (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) and 1.66 g MES hydrate. The pH of the solution was adjusted to 5.9 by adding 1N potassium hydroxide solution. While stirring, 8.4 g Gelzan CM (Sigma-Aldrich Corp. St. Louis, MO USA) were added. The solution was autoclaved (Harvey SterileMax, Thermo Fisher Scientific, Inc., Pittsburg, PA, USA) for 15 min at 121°C and mixed at high speed to enable aeration of the viscous solution before it had a chance to set as a gel.

4.3.3 *S. viridis* seedling inoculation and growth

The *S. viridis* plantlets were inoculated with both *H. seropedicae* RAM4 and *A. brasilense* FP2. The cultures were growth as described above. When the bacterial cultures reached $O.D._{600nm}$ = 1.0 (10^8 cells/mL), the bacteria were washed 3 times with Hoagland's nutrient solution. A mixture of *H. seropedicae* and *A. brasilense* was used as inoculum at a final $O.D._{600nm}$ = 1.0, respectively. The seedlings were inoculated with 1 mL of inoculum per plantlet for 30 min. This bacterial suspension was counted through serial dilutions to confirm the bacterial numbers inoculated. After inoculation, seedlings were transplanted to pots (10 in. x 2.5 in. i.d. tapered cylindrical pot; Stuewe & Son, Inc., Tangent, OR, USA) filled with a 3:1 mix of Turface:vermiculite (Turface was purchased from Profile Products LLC, Buffalo Grove, IL, USA; Vermiculite-A4 course grain was purchased from Whittemore Company, Inc., Lawrence, MA, USA). Plants were grown in Conviron growth chambers (Conviron, Inc., Winnipeg, Manitoba, Canada) set to a 12 h photoperiod at 600 µmol m^{-2} s^{-1} and 22°C. Plants were watered with Hoagland's nutrient solution adjusted for nitrate level of 0.5 mM KNO_3 (for non-inoculated control plants and also for bacterial inoculated plants).

4.3.4 Bacterial counting assay

To evaluate the ability of the bacteria to colonize the selected *S. viridis* genotypes, the roots and leaves were sampled in selected days after inoculation (d.a.i.). In order to count the total number of *H. seropedicae* and *A. brasilense* colonizing the rhizoplane, the roots were washed once and macerated with 1 mL of 0,9% (w/v) NaCl saline solution. In order to evaluated the level of endophytic colonization by *H. seropedicae* counting, the roots and leaves were surface-sterilized with 70% (v/v) ethanol for 40 sec, followed by 1% (w/v) chloramine-T for 40 sec and washed four times in sterile distilled water, before maceration in 0.9% (w/v) NaCl saline solution. Total Root homogenates were serially diluted and plated onto the appropriate selection medium for each bacterium. For *H. seropedicae* selection, the NFbHPN-malate medium containing 80 µg/µL of streptomycin was used. *A. brasilense* selection used the NFb-lactate medium supplemented with 50 mmol/L of phosphate source, 2 mmol/L of NH_4Cl_2, and 80 µg/µL of streptomycin. Colony forming units (CFU) were counted after three days of incubation at 30°C and converted to CFUs per gram of fresh tissue. To ensure that the colonies were from the inoculated bacteria, the colonies were observed under fluorescent stereoscope for *H. seropedicae* or were visualized by staining with 30 mg/mL of X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) for *A. brasilense* (RONCATTO-MACCARI et al., 2003).

4.3.5 $[^{13}N]N_2$ Production

$[^{13}N]N_2$ was produced *via* the $^{14}N(p,pn)^{13}N$ reaction leveraging 18 MeV protons from the BNL Ebco TR-19 Cyclotron (Ebco Industries Ltd, Richmond, BC, Canada). These protons were tightly focused onto the front metal foil of a 50 mL volume gas target leaving 15.5 MeV protons to interact with the gas inside. This target was typically pressurized to 400 psi with 99.9% N_2 + 0.1% O_2. Nitrogen-13 manifests as a by-product during the proton irradiation of this target system which is used at BNL generate $^{11}CO_2$ from the $^{14}N(p,\alpha)^{11}C$ reaction (FERRIERI AND WOLF., 1983). Typically, a 125 µA·min irradiation (25 µA on target for 5 min) would produce 12.95 GBq of $^{11}CO_2$, 4.14 GBq of $[^{14}O/^{15}O]O_2$ and 1.37 GBq of $[^{13}N]N_2$ at the end-of-bombardment. This radioactive gas was immediately processed through a stripping trap (FERRIERI et al., 2005; see Supplemental Figure S1) which was

comprised of 135 mg of silica supported Ni(0) catalyst (Shimadzu, Inc., Kyoto, Japan) mixed with molecular sieve 4 Å (100 mesh; Alltech, Inc., Deerfield, IL, USA). At room temperature this trap removed the majority of the $^{11}CO_2$ component and enabled the remaining components to equilibrate at STP in a 2 L volume tube that was located downstream and inside of a CRC-12 Dose Calibrator (Capintec, Inc, Ramsey, NJ, USA) for direct radiation measurement. At this point, the $^{11}CO_2$ component was reduced substantially to only 148 Bq. Composition of trapped gas was tested using radio gas chromatography analysis (Hewlett Packard 5890 Series; Agilent Technologies, Santa Clara, CA, USA) where components were separated using a spherocarb (100 mesh) packed column (10 ft. x 0.125 in. o.d.; Alltech Associates, Inc., Deerfield, IL, USA).

4.3.6 Tracer Administration

Prior to a $[^{13}N]N_2$ experiment a planting pot containing a single study plant would be sealed into the tracer receiving chamber (see Supplemental Figure S1). This chamber couples to the plastic pot making a gas-tight seal via an imbedded o-ring. A split Plexiglas™ flange is installed around the stem area of the plant and sealed to the pot using tape as a means to isolate the aerial portions of the plant from the belowground portions when tracer was introduced. A small diaphragm pump is affixed to the exhaust line located on this flange and is adjusted to maintain a slight vacuum (~5 Torr below atmosphere). This action ensures that contents of the tracer pulse do not contact the aerial portions of the plant. During pulsing, the contents of the $[^{13}N]N_2$ collection tube are displaced with an air flow of 200 mL min^{-1}. This flow of gas enters the pot through the bottom holes allowing tracer to flow from bottom up through the soil column and exiting through the flange port. A small PIN diode radiation detector (Carroll Ramsey Associates, Inc., Berkeley, CA, USA) positioned on the stem area provides real time feedback of radiation levels during, and after pulsing.

4.3.7 Root Imaging

After exposure to tracer, roots were separated from the Turface:vermiculite plant mix and washed in a PBS solution (x1 strength adjusted to pH 7.4) containing 0.1% Tween 20.

No attempt was made to disentangle the root mass into individual root structures for imaging. Roots were imaged using autoradiography (Typhoon 7000: GE Healthcare, Piscataway, NJ, USA). Images were post-processed using ImageQuant TL 7.0 software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

4.3.8 Radioisotope Decay Analysis

All radioisotopes undergo decay with characteristic properties of radiation release. The radioisotopes that were noted above (^{11}C, $t_{½}$ 20.4 min; ^{13}N, $t_{½}$ 9.97 min; ^{15}O, $t_{½}$ 2.0 min; ^{14}O, $t_{½}$ 77 sec) as being present in the pulse mix all decay by positron emission. However, after annihilation these positrons, regardless of their initial energy, all give rise to the same energy (511 KeV) gamma radiation making them indistinguishable by gamma spectroscopy. The one distinguishing feature of these radioisotopes is they each possess a unique temporal signature for decay. All radioisotopes decay by first-order kinetics according to the following equation where A_0 is defined as the activity at time point zero, A_t as the observed activity at time point t, λ as the decay constant (equal to Log 2/radioactive half-life) and t as the elapsed time:

$$A_0 = A_t\, e^{\lambda t}$$

By virtue of this equation, a plot of the Log A_t versus time will yield a straight line whose slope is the half-life and y-intercept is A_0. For a mixture of radioisotopes multiple lines will manifest from this data treatment enabling the user to extract precise information on isotopic purity.

Tissues targeted for decay analysis were sealed into 10 mL glass vials and placed in a well-type gamma counter where levels of radioactivity were measured every 0.5 min for the duration of at least 1 hr. Decay analysis plots were constructed as described above and linear regression analysis was performed on the data and the fractional radioisotope values calculated. Isotope identification was made on the basis of the calculated half-life from these plots.

Acknowledgements

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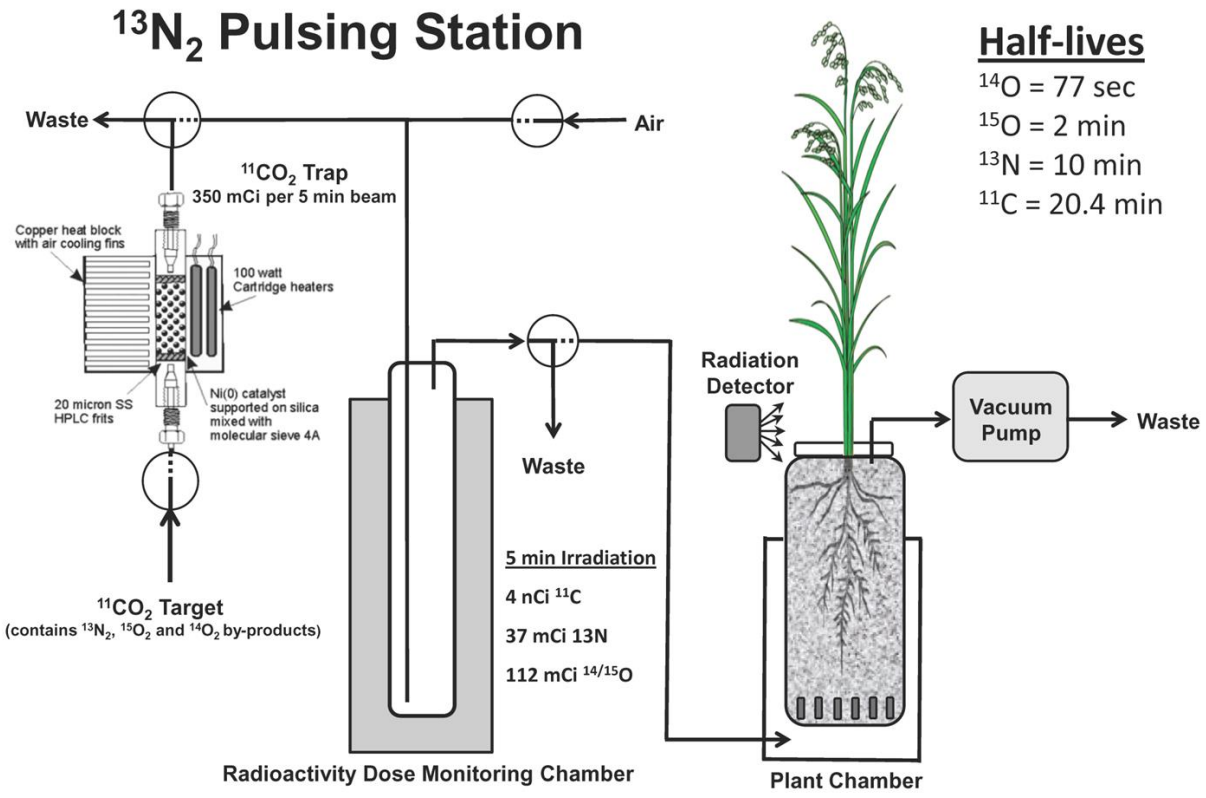
4.4 SUPPLEMENTAL DATA

Supplemental Figure S1: The pulsing station shows the $^{13}N_2$ workflow into the soil column in contact with roots of *Setaria viridis*.



CAPITULO 5

SELEÇÃO DE GENÓTIPOS DE *Brachypodium distachyon* COMO UM POTENCIAL SISTEMA MODELO PARA INTERAÇÃO GRAMÍNEA-BACTÉRIA FIXADORA DE NITROGÊNIO

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Resumo

Bactérias promotoras de crescimento vegetal podem associar – se com gramíneas de importância econômica e agrícola implicando em efeitos benéficos. No entanto, não há um sistema modelo para explorar as respostas de interação planta- bactéria. Neste trabalho, propomos *Brachypodium distachyon*, uma gramínea C_3, como um sistema modelo para estudar as respostas da associação planta-bactéria sob duas condições de nitrogênio. Plântulas de *B.distachyon* foram inoculados três dias após a germinação, com uma mistura de cultura bacteriana contendo *Azospirillum brasilense* e *Herbaspirillum seropedicae*. As plantas foram cultivadas em solo e tratadas em duas condições: (i) sem nitrogênio e, (ii) baixa concentração de nitrogênio (0.5 mM N). Aos 35 dias após a inoculação parâmetros de crescimento (comprimento, número de raízes laterais, massa fresca e seca) foram analisados para raízes e parte aérea. A contagem bacteriana utilizando a raiz das plantas, foi medida utilizando o método de *drop plate* e a colonização das raízes foi visualizada utilizando técnicas de microscopia. Os parâmetros de crescimento mostraram uma ampla variação das respostas das plantas entre os genótipos bem como entre os tratamentos. Respostas de crescimento foram observadas em quatro genótipos cultivados sem adição de nitrogênio e três genótipos tratados com baixo nível de nitrogênio. Ambas as bactérias foram eficientes para colonizar os tecidos radiculares de *B. distachyon*. Em particular, a bactéria endofítica *H. seropedicae* apresentou uma alta colonização no interior das raízes. Os genótipos de *B. distachyon* responderam diferencialmente a promoção de crescimento, independente da condição de nitrogênio, quando inoculadas com *A. brasilense* e *H. seropedicae*. Estes dados sugerem que *Brachypodium distachyon* pode ser utilizado como um sistema modelo para estudar o processo de colonização entre gramínea – bactéria, já que demonstrou uma forte associação bacteriana, mesmo não demonstrando efeitos de promoção de crescimento.

5 SCREENING OF *Brachypodium distachyon* AS A POTENTIAL MODEL GRASS FOR BACTERIAL INTERACTION

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Abstract

Background and aims Plant growth promotion bacteria can associate with important crop grasses given beneficial effects. However there is no model system to explore the responses of plant-bacteria interaction. In this work we propose *Brachypodium distachyon* as a plant model system to study the responses of plant-bacteria association under two nitrogen conditions.

Methods B.distachyon seedlings were inoculated three days after germination with a bacterial culture combined with *Azospirillum brasilense* and *Herbaspirillum seropedicae*. Plants were grown in soil under with no nitrogen and low nitrogen condition. At 35 days after inoculation growth parameters (length, number of lateral roots, fresh and dry weight) were analyzed to roots and shoots. The root bacterial counting was measured by drop plate method and the root colonization was accomplished by microscopy techniques.

Results The growth parameters showed a wide range variation of responses among the genotypes and treatments. The positive growth responses was observed in four genotypes grown under no nitrogen and three genotypes treated with low nitrogen level. Both bacteria were efficient to colonize *B. distachyon* root tissues. In particular the endophytic bacteria *H. seropedicae* that showed a strong colonization inside the roots tissues.

Conclusions The *B. distachyon* genotypes responded differentially to plant growth promotion even in the same treatment when inoculated with both bacteria *A. brasilense* and *H. seropedicae*. These data thus suggest that *Brachypodium distachyon* may not been a good system model to analyze the plant growth promotion effects but can be used as a model to clarify the colonization process since have demonstrate a strong bacterial association.

Keywords Plant model system *Brachypodium distachyon.* Plant-bacteria interaction. *Azospirillum brasilense. Herbaspirillum seropedicae.* Plant growth promotion.

5.1 INTRODUCTION

Grasses provide, directly (cereals) or indirectly (animal feed), the bulk of human nutrition and are thus essential in addressing the strategic goal of food security. In addition, grasses are also poised to become major sources of renewable energy. However the agricultural system is highly dependent on chemicals fertilizers inputs to provide nutrients for plant development and also maintain agricultural productivity in terms of yield. The high use of chemical fertilizers can have adverse effects on environment including water contamination and less plentiful soil as well. For conservation reasons becomes essential to explore strategies to minimize the chemicals usage keeping the productivity. One of strategy is to exploit the potential of plant beneficial microbes as crops inoculants (OKON & LABANDERA-GONZALEZ, 1994), include plant growth promotion rhizobacteria (PGPR). The PGPR can associate with root system of several important crops as rice (*Oryza sativa*), maize (*Zea mays*), sorghum (*Sorghum bicolor*) and also sugarcane (*Saccharum* sp) promoting plant growth in different ways as associative nitrogen fixation, production phytohormones and others signals. It is the case of *Azospirillum brasilense* a diazotrophic bacterium that is able to colonize the external tissue and has been used as crops inoculants on field. In maize, a combination of *Azospirillum* and *Pseudomonas* can enhance plant-beneficial properties (COMBES-MEYNET et al., 2011), the co-inoculation becoming a promising plant-beneficial strategy. Also *Herbaspirillum seropedicae,* an endophytic bacteria, which is able to colonize the internal tissues of plants promoting growth. The root colonization by this bacteria has been elucidated (MONTEIRO et al., 2012) as well as sequenced genome (PEDROSA et al., 2011). These bacteria are able to establish association with members of Poaceae family often in high numbers, without damage and without eliciting symptoms of plant disease (REINHOLD-HUREK & HUREK, 2011).

The members of family Poaceae are excellent models to study the interaction of cereal crops with diazotrophic bacteria. Lots of works have shown the successful of interaction with rice, sugarcane, wheat and maize and also the positive effects as plant growth promotion and nitrogen fixation. However there is no model system for these studies in grasses, such as *Arabidopsis*, that is the best plant model but is far from Poaceae family. The major research to study the response of plant-bacteria interaction usually takes rice and maize. Even those plants have been a good model to functional genomics studies, they are hard handling because of the size and long life cycle.

Brachypodium distachyon is an annual C_3 grass and has been proposed as a new model species for grasses because it possesses many of the characteristics required for a tractable model system, including small stature (20 cm), a compact genome size (272 Mb), a short life cycle, self-fertility, the availability of a complete genome sequence, ease of transformation and extensive natural variation in biological traits (BEVAN, GARVIN E VOGEL, 2010). Thus, significant investments have been made in developing and using *Brachypodium* as a model for these emerging biofuel crops (BRKLJACIC et al., 2011).

In the present work, we investigated the plant responses of *B. distachyon*, as a potential model grass, in interaction with two well characterized diazotrophic bacteria, *A. brasilense* and *H.seropedicae* under no nitrogen and low nitrogen (0.5mmol N) source relative to control plants. To evaluate the responses of *B.distachyon* we analyzed growth parameters on roots and shoots and the colonization was measured by microscopy.

5.2 MATERIAL AND METHODS

5.2.1 Bacterial strains and media

In this study two diazotrophic bacterial strains were used: *Herbaspirillum seropedicae* strain RAM4 and *Azospirillum brasilense* strain FP2-7 both gently provide by Dr. Fabio Pedrosa, the Federal University of Parana - Curitiba - Brazil. The bacteria were routinely grown in an orbital shaker at 30◦C (120 rpm) in NFbHPN medium (HP means high phosphate). For *H. seropedicae* medium was supplemented with 20% potassium malate (KLASSEN et al., 1997) and *A. brasilense* was supplied with 50% potassium lactate (MACHADO et al., 1991). The nitrogen source was used ammonium chloride (NH_4Cl) for both bacteria.

5.2.2 Plant material

Several accessions of *B. distachyon* were surface sterilized by immersion in 6% bleach plus Tween-20 (USB, Cleveland, OH, USA) solution for 3 min, and subsequently washed 5 times with sterile distilled water. Treated seeds were placed on a disc of filter paper,

moistened with 1 mL Hoagland in a sealed Petri dish and kept at 4◦C for 1 week to synchronize germination. After vernalization the seeds were germinated on plates containing 1% phytagel plus Hoagland's solution without nitrogen source, supplied with gibberellic acid (1μM) and maintained 3 to 5 days in growth chamber at 30◦C in the dark.

5.2.3 Inoculation and growth conditions

After germination the uniform seedlings were selected and inoculated in sterile plates containing 1mL of washed bacterial cultures per seedling ($O.D_{600}$ =1; 10^7 cells mL^{-1}) diluted in sterile Hoagland solution without nitrogen source. The seedlings were inoculated with a combination of *H. seropedicae* strain Ram4 and *A. brasilense* strain FP2-7 for 30 min. After that seedlings were transplanted to soil containing a mixture of sterile turface and vermiculite (3:1). Plants were grown under two conditions: 1) without nitrogen addition and 2) low nitrogen (0.5mM) source, according to the number of germinated seeds. Plants were randomly placed in a growth chamber for 35 days, under controlled conditions (20 h light: 4 h dark photoperiod with cool-white fluorescent lighting at a level of 150 μEm-2s-1). The plants were irrigated daily with distillated water and twice a week with 30 mL of Hoagland´s nutrient solution. Seedlings inoculated with boiled killed bacteria were used as control and were grown in the same conditions of inoculated.

5.2.4 Analysis on plants

During plant development several parameters were analyze such as number of leaf development, number of nodes, number of tiller detectable and also time of flowering. At 35 days after inoculation (dai), plants were harvested and carefully removed from soil and washed briefly with distillated water and then they were analyzed root length and number of lateral roots using WinRHIZO 2002c (Régent Instrument Inc., Québec City, Canada). In parallel, parameters shoot length, root and shoot fresh and dry weights, were measured.

5.2.5 Analysis of bacterial root colonization by microscopy

Herbaspirillum seropedicae strain RAM4 is a DsRed-tagged recombinant colony, which produced a reddish color (MONTEIRO et al., 2008). To investigate the colonization by bacteria, roots from 2 to 4 plants with 35 days after inoculation were collected and cut through two different root zones: upper (zone of differentiation) and lower (zone of elongation). Dissected root fragments from control and inoculated plants were placed in a drop of water, coverlipped and observed under a fluorescence microscope (Olympus IX70 inverted microscope) using the ET-DSRed 590-650 nm bandpass filter. For confocal microscopy, the roots were cut as described above and observed using a Zeiss LSM 510 META laser scanning confocal microscope quipped with 488 nm argon and 543 nm He–Ne lasers to detect green fluorescence emitted by a GFP-tagged *H. seropedicae* (excitation at 488 nm and detection at 500-550 nm) and red fluorescence emitted by DsRed-tagged bacteria of the RAM4 strain (excitation at 543 nm and detection at 488-633). For better localization of the bacteria in the internal root tissue some of the root genotypes were incubated with 2 μM of propidium iodide (PI) for 5 minutes and washed 5 times with DI water. PI staining was visualized with the argon laser (excitation at 488 nm, detection at 565–615 nm). When PI staining was performed, images of red PI fluorescence were overlaid with the images of the GFP-tagged bacteria and with brightfield transmitted light images of the root parts. Images red fluorescence from DsRed-tagged bacteria were overlaid with the transmitted images (brightfield mode) of the root parts. All composite images were produced using the LSM Image Browser 4.0 software (Carl Zeiss Microimaging).

5.2.6 Statically analysis

The statistical analysis was performed through non parametric *Wilcoxon test* ($p \leq 0.05$) using R software v2.15.3. Different numbers of plants were analyzed for each genotype according to the number of germinated seeds.

5.3 RESULTS

5.3.1 *Brachypodium distachyon* screening

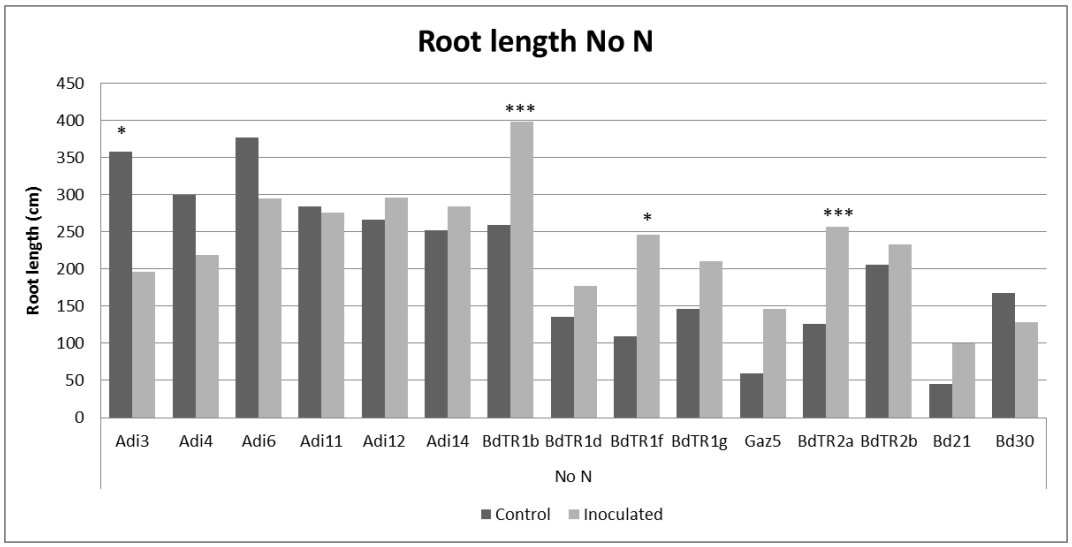
Fourth three accessions of *B. distachyon* were inoculated with a culture mix of both bacteria and the development of plants was analyzed weekly. Among those accessions, some of them presented low germination rate or did not germinated, symptoms of deficiency nutrition also difficult adaptation to laboratory conditions and died on the first week after transplantation. At least 23 accessions were analyzed as number of leaves, nodes and number of tillers in two different conditions, under no nitrogen and under low nitrogen condition that was supplied with 0.5mmol of KNO_3.

5.3.2 Plant growth promotion

These bacteria have been known to be able to colonize important grasses and also work as plant growth promoter. Such plant growth-promoting bacteria (PGPB) or plant growth promoting rhizobacteria (PGPR) can stimulate plant growth, increase yield, reduce pathogen infection, as well as reduce biotic or abiotic plant stress (BOTTA et al., 2013). Initially, we studied the response of plant to bacteria interaction through roots and shoots analysis 35 days after inoculation. Parameters as root and shoot length, number of lateral roots, roots and shoots fresh and dry weight were measured. The accessions of *Brachypodium distachyon* were treated under no nitrogen (no N) and low nitrogen (low N) condition according with the number of germinated seeds.

Among fifteen accessions of *B. distachyon* treated under no nitrogen condition, inoculated plants of genotypes BdTR1b, BdTR1f and BdTR2a presented significant difference for root length relative to control plants. Although there was no significant difference, some of inoculated genotypes showed a slight trend to increase, when compared with control samples such as BdTR1d, BdTR1g, Gaz5 and Bd21. However, Adi3 was more significant on control plants than inoculated. In addition Adi4 and Adi6 showed an increase response on control plants as well, though no significant (Figure 5.1).

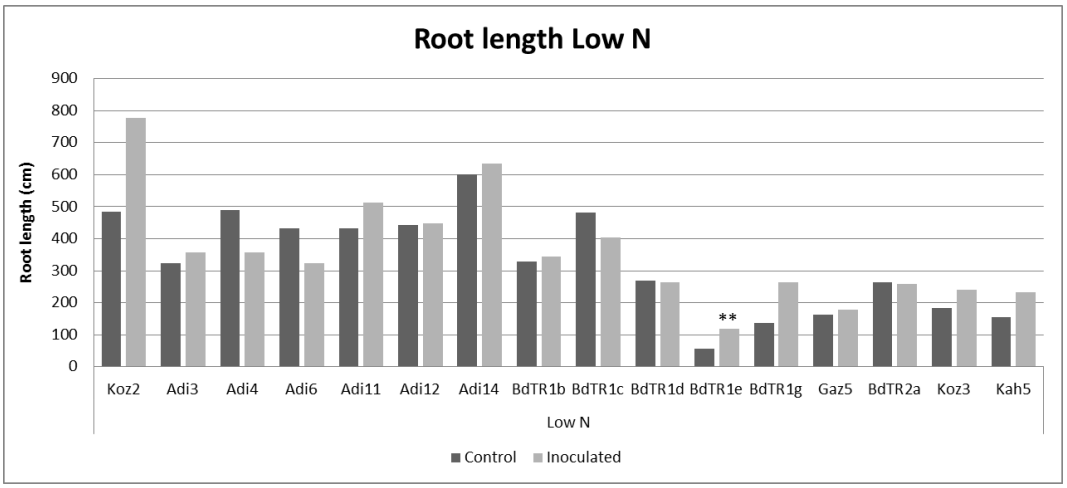
Figure 5.1. Root length (cm) in control and inoculated roots of *Brachypodium distachyon* under no nitrogen condition. The growth parameter was measured in plants with 35 days after inoculation with *Azospirillum brasilense* and *Herbaspirillum seropedicae*. Among fifteen genotypes treated under no nitrogen condition three inoculated (BdTR1b, BdTR1f and BdTR2a) showed positive responses to inoculation relative to control plants. Bars indicate means ±SE and asterisks indicate the significance difference (*P≤0.05, **P≤0.01, ***P≤0.001).



Plants under low nitrogen condition, there was only one genotype, BdTR1e, with significant increase on inoculated. The major of control and inoculated samples showed a similar response for root length, even Koz2 that seems present a rise relative to control, though not significant (Figure 5.2).

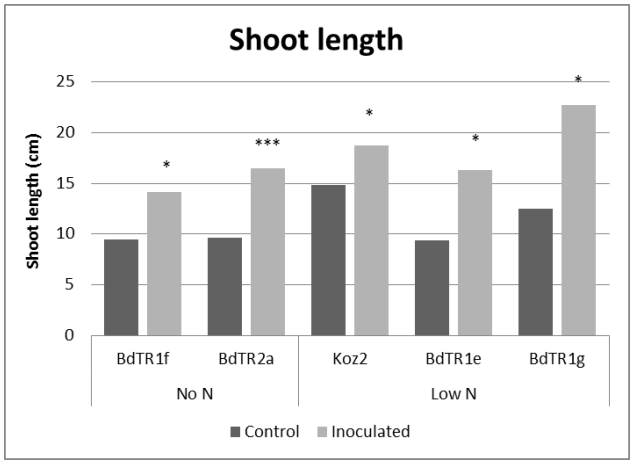
Since *H. seropedicae* is able to colonize internal tissues and spread from roots to shoot through xylem, we also evaluated the effects on shoot length. Plants grown without nitrogen source, BdTR1f and BdTR2a of inoculated seedlings were increased. At least three genotypes, Koz2, BdTR1e and also BdTR1g presented a significant growth on shoot length in inoculated plants treated with low nitrogen (Figure 5.3).

Figure 5.2. Root length (cm) in control and inoculated roots of *Brachypodium distachyon* under low nitrogen condition. The growth parameter was measured in plants with 35 days after inoculation with *Azospirillum brasilense* and *Herbaspirillum seropedicae*. Among sixteen genotypes treated under low nitrogen condition only one inoculated (BdTR1e) showed positive responses to inoculation relative to control plants. Bars indicate means ±SE and asterisks indicate the significance difference (*P≤0.05, **P≤0.01, ***P≤0.001).



A. brasilense produces beneficial effects on the host plant, particularly on the roots area. It is well known that the association between diazotrophic bacteria and grasses leads to, amongst other benefits, increased lateral root development (MANTELIN AND TOURAINE 2004; KROME et al., 2009).

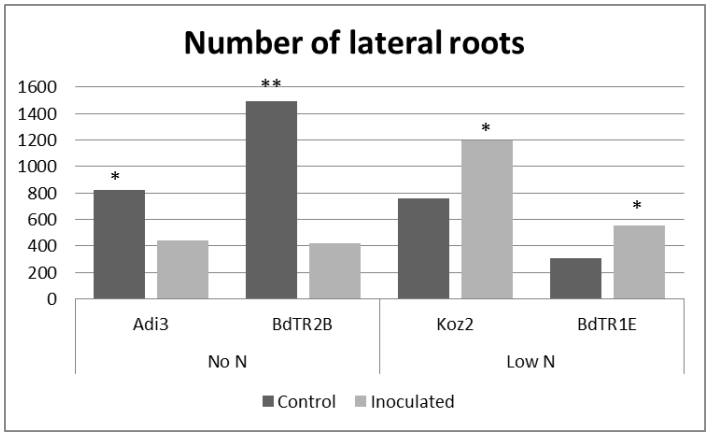
Figure 5.3. Shoot length (cm) in control and inoculated roots of *Brachypodium distachyon* under no and low nitrogen condition. The growth parameter was measured in plants with 35 days after inoculation with *Azospirillum brasilense* and *Herbaspirillum seropedicae*. Under no nitrogen condition two inoculated genotypes (BdTR1f and BdTR2a) showed significant difference. Under low nitrogen condition three genotypes (Koz2, BdTR1e and BdTR1g) showed significant difference to inoculation relative to control plants. Bars indicate means ±SE and asterisks indicate the significance difference (*P≤0.05, **P≤0.01, ***P≤0.001).



Number of lateral roots also was measured for all of plants, therefore Koz2 and BdTR1e inoculated responded positively under low nitrogen condition. Interestingly, under no nitrogen condition the controls Adi3 and also BdTR2b had an increase on lateral roots

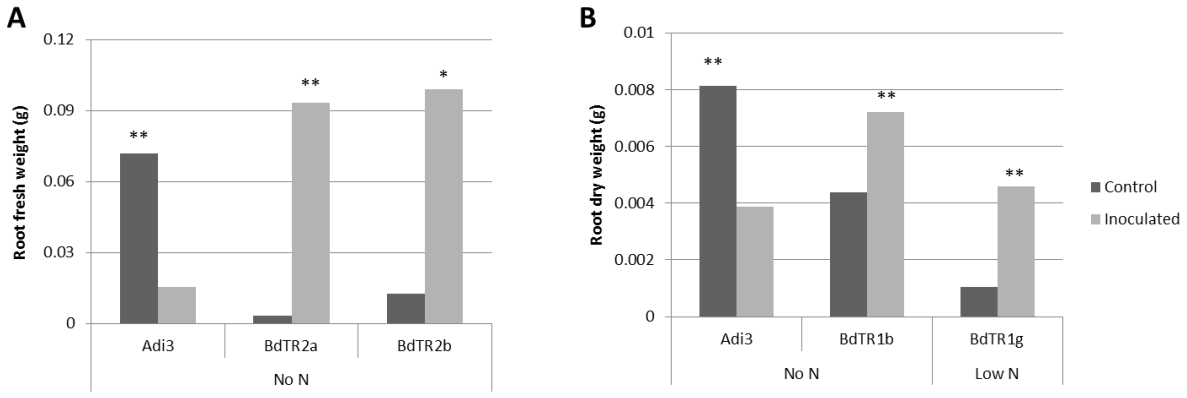
number (Figure 5.4), that we hypothesized could be an abiotic stress response since those plants are looking for nutrients to sustain their growth.

Figure 5.4. Number of lateral roots in control and inoculated roots of *Brachypodium distachyon* under no and low nitrogen condition. The growth parameter was measured in plants with 35 days after inoculation with *Azospirillum brasilense* and *Herbaspirillum seropedicae*. Under no nitrogen condition only control plants (Adi3 and BdTR2b) showed significant difference. Under low nitrogen condition three genotypes (Koz2 and BdTR1e) showed significant difference to inoculation relative to control plants. Bars indicate means ±SE and asterisks indicate the significance difference (*P≤0.05, **P≤0.01, ***P≤0.001).



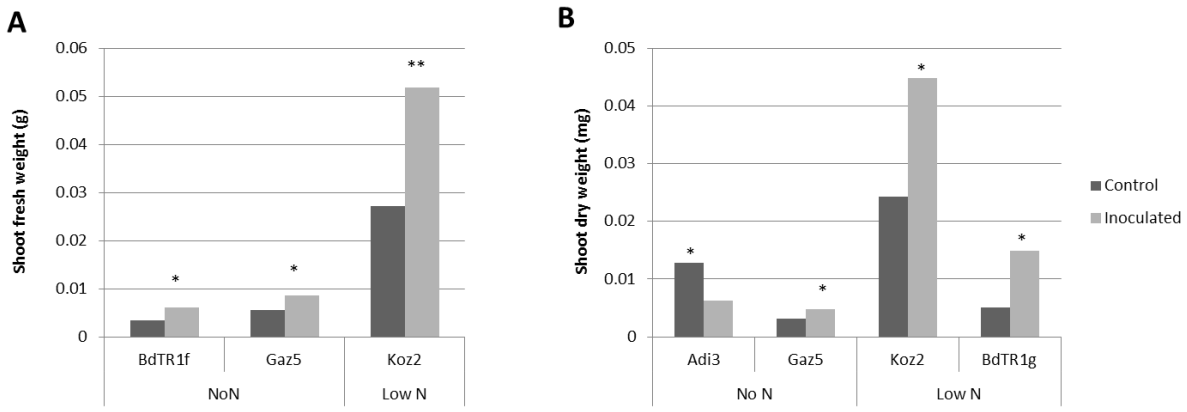
In addition, inoculated plants under no nitrogen condition showed increase at root fresh weight in three genotypes relative to control plants (Figure 5.5A). There was no significant response in the plants treated under low nitrogen for that parameter. In the other way to root dry weight, Adi3 (control) and BdTR1b (inoculated) under no nitrogen presented an increase as well BdTR1g (inoculated) under low nitrogen condition (Figure 5.5B).

Figure 5.5. Root fresh and dry weight (g) were measured in control and inoculated plants of *Brachypodium distachyon*, 35 days after inoculation with *A. brasilense* and *H.seropedicae*. A: Root fresh weight (g) in plants of *B. distachyon*, grown under no nitrogen condition, two genotypes (BdTR2a and BdTR2b) showed significant difference to inoculation relative to control plants. There was no response in plants treated under low nitrogen condition for this parameter. B: Root dry weight (g) in plants of *B. distachyon*, grown under no nitrogen condition, two genotypes Adi3 (control) and BdTR1b (inoculated) showed significant difference. BdTR1g treated under low nitrogen condition showed significant difference to inoculation relative to control plants. Bars indicate means ±SE and asterisks indicate the significance difference (*P≤0.05, **P≤0.01, ***P≤0.001).



Additionally, two accession that grown with bacterial mix without nitrogen showed an increased on shoot fresh weight as well koz2 inoculated low nitrogen plants (Figure 5.6A). Shoot dry weight was measured and an increase was observed in Gaz5 under no nitrogen and also BdTR1g and Koz2 under low nitrogen condition in inoculated plants (Figure 5.6B). Interestingly, Adi3 without nitrate showed increase in control plants for root length, root and shoot dry and also fresh weight.

Figure 5.6. Shoot fresh and dry weight (g) were measured in control and inoculated plants of *Brachypodium distachyon*, 35 days after inoculation with *A. brasilense* and *H.seropedicae.* A: Shoot fresh weight (g) in plants of *B. distachyon*, grown under no nitrogen condition, two genotypes (BdTR1f and Gaz5) showed significant difference to inoculation relative to control plants. Koz2 grown under low nitrogen condition, also showed significant difference to inoculation relative to control plants. B: Shoot dry weight (g) in plants of *B. distachyon*, grown under no nitrogen condition, two genotypes Adi3 (control) and Gaz5 (inoculated) showed significant difference. Koz2 and BdTR1g, plants grown under low nitrogen condition, showed significant difference to inoculation relative to control plants. Bars indicate means ±SE and asterisks indicate the significance difference (*P≤0.05, **P≤0.01, ***P≤0.001).

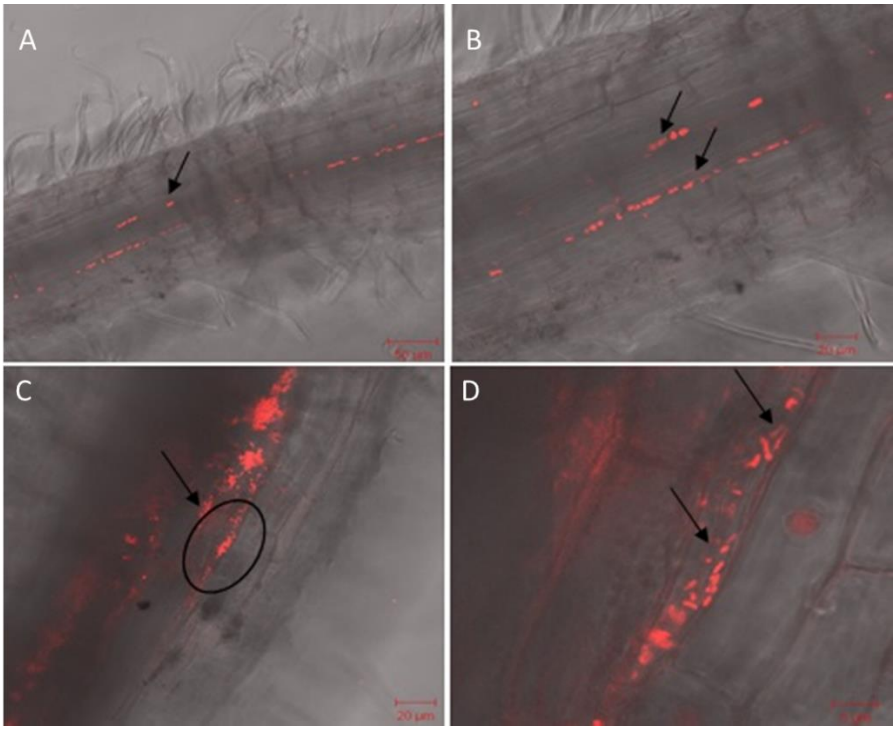


5.3.3 Rhizosphere and internal colonization by *Herbaspirillum seropedicae* SmR1 and *Azospirillum brasilense nifH:gusA* expression

It is well known that *Herbaspirillum seropedicae* attachment starting to roots surfaces and the subsequent colonization of the emergence points of lateral roots and then spreading through intercellular space also entry by xylem roots via lateral roots cracks (LRC) in maize and rice plants (MONTEIRO et al., 2012). Using microscopic tools, which allow the detection of *Dsred*-labelled strains by fluorescence, we captured image from *Herbaspirillum seropedicae.* Plants 35 days old were used for microscopy analysis, removed from soil, were washed and cut in two roots zones. The most of genotypes presented bacterial cells attached on the surface to the roots as a single cells or even forming microcolonies. We observed that

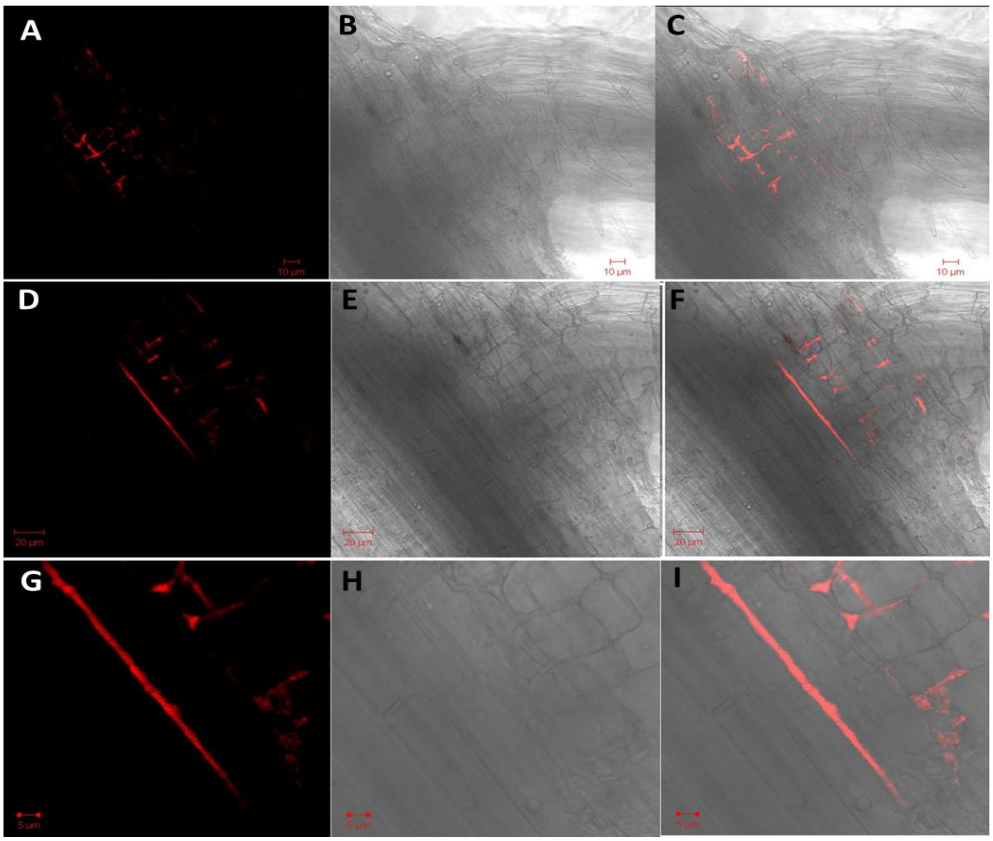
plants under low nitrogen condition have shown a strong surface colonization such as BdTR1c (data not shown). In this genotype the bacterium mainly colonized the elongation zone and migrates through intercellular spaces to reach vegetative plants parts. As Koz3 the bacteria colonization is located on the bottom of lateral roots cracks at differentiation zone even 35 days after inoculation. A low density of bacterial cells has been detected in inoculated plants under no nitrogen condition although Gaz5 and Bd21 demonstrated a strong colonization without nitrogen (Figure 5.7A-D).

Figure 5.7. Roots of *Brachypodium distachyon* genotype Bd21, inoculated with *H. seropedicae* (*DsRed* triggered) grown under no nitrogen condition. Roots were observed at 35 days after inoculation using a confocal microscope. A: Intercellular spaces colonized by *H. seropedicae* observed from root elongation zone in Bd21 genotype. B: A magnification of the region in A indicate by the arrow. C: High colonization of *Herbaspirillum seropedicae* in the intercellular spaces observed in genotype Bd21 at root elongation zone. D: A magnification of the highlighted region in C indicate by the arrow that show the microcolonies of *H. seropedicae.*



Bd21 was analyzed by confocal microscope which allowed seeing a high density of *H. seropedicae* on roots surface as well as endophytic colonization (Figure 5.8A-I). All plants analyzed from Bd21 presented high bacterial cells close of lateral roots in the differentiation zone forming colonies, however, each genotype that was analyzed showed a different pattern of colonization. It has been known the plant association depends of plant genotype and bacteria strain (REINHOLD-HUREK E HUREK, 2011; MONTEIRO et al., 2012; VARGAS et al., 2012).

Figure 5.8. Roots of *Brachypodium distachyon* genotype Bd21, inoculated with *H. seropedicae* (*DsRed* triggered) grown under no nitrogen condition. Roots were observed at 35 days after inoculation using a confocal microscope. A: Intercellular spaces colonized by *H. seropedicae* observed from root elongation zone close to lateral roots in Bd21 genotype. B: Brightfield image mode. C: Overlay mode shown the bacteria located under lateral roots zone. D and G: Are a magnification of the region A. E and H: Brightfield image mode. F and I: Overlay mode shown the bacteria located under lateral root region, colonized intercellular spaces of Bd21 roots.



To identify *Azospirllum brasilense*, colonization assay was performed using drop plate method with NFb lactate medium with 0.5mg/ml of x-gluc. After 48h the blue spots were counting presented about 10^2 to 10^6 UFC/g of fresh tissue after 35 days pos inoculation. Roots were incubated in Cacodylate buffer with X-gluc, however, was not possible express the *gus* on *B. distachyon* roots.

5.4 DISCUSSION

In this work we screened 23 genotypes of *Brachypodium distachyon* inoculated with two diazotrophic bacteria to identify the effects of plant growth and colonization. Since these bacteria are capable to fixing nitrogen, we grew the plants under no nitrogen and low nitrogen conditions. The analysis of growth parameters showed a wide range variation among the

genotypes in both nitrate condition. It has been known that associative beneficial bacteria can increase their host growth, however in particular cases despite the optimistic expectations on the impact of associative diazotrophic nitrogen fixation, most inoculation experiments did not show substantial contribution to plant growth (DOBBELAERE et al., 2003). As the diazotrophic microorganisms possess different mechanisms of plant growth promotion the nutrient uptake is the net result of numerous ways of action that may initiated simultaneously (RICHARDSON et al., 2009).

Brachypodium distachyon plants showed a strong colonization by *Herbaspirillum seropedicae* even after 35 days pos inoculation. The ability of this bacteria to colonize internal tissues of grasses have been clarified in maize plants by Monteiro et al (2012). The majority of the bacteria remain on the root surfaces, but some herbaspirilla can penetrate through discontinuities of the epidermis, such as the elongation zone and lateral root cracks (LRCs) and then a rapid occupation of root intercellular spaces. *H. seropedicae* is also able to move from the roots to the aerial parts in the xylem colonizing the shoots and leaves (MONTEIRO et al., 2012). The bacterial endophytic microbiome promotes plant growth and health and beneficial effects are in many cases mediated and characterized by metabolic interactions. Recent advances have been made in regard to metabolite production by plant microsymbionts showing that they may produce a range of different types of metabolites. These substances play a role in defense and competition, but may also be needed for specific interaction and communication with the plant host (BRADER et al., 2014). The *B. distachyon* genotypes responded differentially to plant growth promotion even in the same treatment when inoculated with both bacteria *A. brasilense* and *H. seropedicae*. These data thus suggest that *Brachypodium distachyon* may not been a good system model to analyze the plant growth promotion effects but can be used as a model to clarify the colonization process since have demonstrate a strong bacterial association.

Acknowledgements

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CONSIDERAÇÕES FINAIS

A interação entre gramíneas e bactérias diazotróficas tornou-se uma estratégia importante para obtenção de nitrogênio, através da FBN, bem como estimular o crescimento e o rendimento de plantas. Embora, bactérias como *Herbaspirillum seropedicae* e *Azospirillum brasilense* sejam bem caracterizadas, as modificações causadas na planta pela interação ainda nao estão totalmente elucidadas.

No presente trabalho foi possivel observar a influência da bactéria endofítica *H. seropedicae* em plântulas de milho cultivadas em areia. Plantas inoculadas apresentaram um aumento no número de raízes laterais bem como um aumento no nível de transcritos de genes envolvidos na sinalização (*Zmko1*) e defesa (*Zmrboh C*) das plantas. Do mesmo modo, parâmetros de crescimento foram analisados em sistemas modelos como *Brachypodium distachyon* e *Setaria viridis*, quando inoculados com *A. brasilense e H. seropedicae*. *Brachypodium distachyon* demonstrou uma grande variação nas respostas aos parâmetros de crescimento, porém pode ser considerado um modelo para estudos relacionados ao processo de colonização e interação planta-bactéria devido à alta colonização por ambas bactérias, em particular por *H. seropedicae.*

Radioisótopos como Carbono-11 ($^{11}CO_2$) e Nitrogênio-13 ($^{13}N_2$) foram administrados em plantas inteiras de *Setaria viridis* inoculadas com as bactérias fixadoras de nitrogênio *A. brasilense e H. seropedicae*. A utilização de $^{11}CO_2$ permitiu analisar respostas fisiológicas como porcentagem de carbono fixado e fotassimilados bem como respostas metabólicas (perfil de açúcares) da planta. O uso de $^{13}N_2$ em plantas de *S. viridis* demonstrou evidências da incorporação de nitrogênio fixado, nas plantas inoculadas com *A. brasilense* e *H.seropedicae*.

O uso de bactérias benéficas associativas com gramíneas, principalmente com cereais, tem sido de grande importância para melhorar o crescimento das plantas, minimizando o uso de produtos químicos. Neste trabalho, mostramos a influência de bactérias capazes de fixar nitrogênio e causar modificações fisiológicas e metabólicas em diferentes espécies de gramíneas. As informações geradas servirão de base para futuras pesquisas utilizando bactérias promotoras de crescimento vegetal.

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